# SALT OPTIMA IN AGGLUTINATION

by

A. E. PLATT[1]

(From the Department of Bacteriology, University of Adelaide, and the Institute of Medical and Veterinary Science, Adelaide.

*(Submitted for publication 1st September, 1938.)*

In the course of a study of the effects of salt concentration on the antibody and antigen optima in agglutination with a *Brucella abortus* suspension and the homologous antiserum, it was observed that effects similar to those described by Bier (1931) and Duncan (1934) were not demonstrable. In consequence it became necessary to enlarge the scope of this portion of the work, and the results obtained are recorded in this paper.

## HISTORICAL.

Using a Flexner strain of dysentery bacillus and homologous antiserum, Bier *(loc. cit.)* showed that, with increasing concentrations of salt, there was an increase in the antibody-antigen ratio in the optimum mixture. He explained his results in terms of the hypothesis expressed by Northrop and de Kruif (1922 a, b) that in addition to reducing the difference of potential between the organisms, salt also decreased the cohesive force between them.

Duncan *(loc. cit.)* using *Bact. flexneri* confirmed these findings and showed that, over a wide range of salt concentrations, the ratio of antibody to antigen varied from a high ratio with normal salt concentration to a very low ratio with low concentrations of electrolyte. In addition he showed that the salt concentration also influenced the end point of complete agglutination.

In a later paper Duncan (1937) determined the salt optima for a number of antigen-antibody mixtures and showed that the position of the salt optimum was independent of the ratio of antigen to antibody, but that it was directly influenced by the concentration of antiserum present in the mixture.

## EXPERIMENTAL WORK.

*Suspension.*

The organism used, isolated two months previously from an infected foetus (calf), was perfectly salt-stable (Pandit and Wilson, 1932), and still required $CO_2$ for growth. It was grown

[1] Aided by a grant from the Commonwealth Grant to the University of Adelaide.

for three days on liver-extract-agar (Holth, 1911) in an atmosphere of 10 p.c. $CO_2$ in air at 37°C.; the resultant growth was then washed off in distilled water and killed by heating at 55°C. for thirty minutes. The organisms were recovered by centrifugation and re-suspended in distilled water and dialysed in the ice-box until absolutely free from salt. The suspension was stored in the cold at a concentration of $40 \times 10^9$ organisms per ml.

*Antiserum.*

This was obtained from an infected cow and was dialysed against distilled water until salt-free. The precipitate which formed was removed by centrifugation. No preservative was added, nor was the serum heated, but, as with the suspension, was stored in the cold. When titrated in an ordinary agglutination reaction against the suspension diluted to contain 1,000 million organisms per ml., the dialysed serum gave complete agglutination in a dilution of 1 : 5,120.

*Salt Solution.*

Pure sodium chloride was made up to a 4 molar concentration in doubly distilled water (from glass still) and will be referred to throughout this paper as "salt".

*Technique.*

For all experiments involving determination of an optimum the reagents were placed in Monax glass tubes 8 mm. × 120 mm. and incubated in an illuminated water bath at 52°C. Each tube contained 0·5 ml. of bacterial suspension made up to double the desired concentration in distilled water, 1 ml. salt solution at double the desired concentration, and 0·5 ml. of half the desired dilution of antiserum. When mixed the desired concentration of each reagent was obtained. In constant antibody titrations the antigen was added first, followed by the salt and finally the antiserum. In constant-antigen titrations the reverse procedure was adopted.

*Determination of Antibody and Antigen Optima.*

"Coarse" titrations in which two-fold dilutions of the variable reagent were used showed that, for salt concentrations from 2M/7 to M/56, the antibody and antigen optima remained constant. No agglutination occurred with a salt concentration of M/112 and concentrations in excess of 2M/7 were not used. In "fine" titrations it was found that the ratio of the number of organisms to 1 ml. antiserum was $2 \times 10^{12}$ in the *antigen* optimum and $7 \times 10^{11}$ in the *antibody* optimum.

At least in so far as the antibody optimum is concerned these results are not in accord with those of Duncan who found, in the same range of salt concentrations, that the shift in the optimum was proportional to the degree of variation in the salt concentration.

*Determination of the Salt Optimum for Antigen-Antibody Mixtures.*

A range of concentrations of bacterial suspension was put up against a number of dilutions of antiserum. The numbers of organisms put up against any single dilution of antiserum were so arranged that *antigen* and *antibody* optimal numbers were included, and also mixtures containing numbers of organisms in excess of and

less than these two. The salt optimum for each combination was determined. The results are given in Table 1.

TABLE 1.

*Salt Optima Determined for a Combination of Br. Abortus Suspension and Br. Abortus Antiserum (Both Dialysed).*

| Suspension No. organisms per ml. × $10^9$. | Anti-serum dilutions. | Ratio No. organisms to 1 ml. anti-serum × $10^9$. | Salt Concentration. 4M/7 | 2M/7 | M/7 | M/14 | M/28 | M/56 | M/112 | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|
| 20·000 | 1/175 | 3,500 | | | | + | | | | |
| 11·428 | „ | 2,000 | | | | + | | | | Constant antibody O.R. |
| 5·714 | „ | 1,000 | | | | + | | | | |
| 4·000 | „ | 700 | | | | + | | | | Constant antigen O.R. |
| 2·000 | „ | 350 | | | | + | | | | |
| 1·000 | „ | 175 | | + | —— | + | | | | |
| 0·500 | „ | 87·5 | | + | | | | | | |
| 11·428 | 1/350 | 4,000 | | | | + | | | | |
| 5·714 | „ | 2,000 | | | | + | | | | Constant antibody O.R. |
| 2·000 | „ | 700 | | | | + | | | | Constant antigen O.R. |
| 1·000 | „ | 350 | | | + | | | | | |
| 0·500 | „ | 175 | | + | —— | + | | | | |
| 5·714 | 1/700 | 4,000 | | | + | | | | | |
| 2·857 | „ | 2,000 | | | | + | | | | Constant antibody O.R. |
| 2·000 | „ | 1,400 | | | | | + | | | |
| 1·000 | „ | 700 | | | | | | + | | Constant antigen O.R. |
| 0·500 | „ | 350 | | | | + | | | | |
| 2·857 | 1/140 | 4,000 | | | + | | | | | |
| 1·4285 | „ | 2,000 | | | + | —— | + | | | Constant antibody O.R. |
| 1·000 | „ | 1,400 | | | | | + | | | |
| 0·500 | „ | 700 | | | | | + | | | Constant antigen O.R. |
| 0·250 | „ | 350 | No agglutination in 24 hours | | | | | | | |
| 0·71425 | 1/2800 | 2,000 | Zone of agglutination* | | | | | | | Constant antibody O.R. |
| 0·250 | „ | 700 | No agglutination in 24 hours | | | | | | | Constant antigen O.R. |

* Optimum at salt concentration greater than 8M/7 (see text).
A + sign indicates position of optimal agglutination.

It will be observed that with bacterial suspensions containing $2 \times 10^9$ organisms per ml. or more, and with serum dilutions of 1/350 or lower, the salt optimum is constant at M/14 salt. Outside these ranges there is a marked shift in the optimum for most of the mixtures. It will be observed that the salt optimum remained constant at M/14 for the constant-antibody optimal mixtures for dilutions of antiserum up to 1/1400 but with higher dilution of the serum it moved to the left to a very much greater salt concentration. The constant antigen optimal

mixtures, however, behaved quite differently, in that at an antiserum dilution of 1/700 there was a marked movement to the right to as low a salt concentration as M/56. At a 1/1400 serum dilution it had moved up to M/28 salt. No salt optimum was obtained at serum dilution of 1/2800. With the constant antibody system the appearance of agglutination first in a 8M/7 salt concentration only after a very prolonged incubation probably indicated that the optimal salt concentration lay above 8M/7. No agglutination was observed in any of the constant antigen mixture tubes at this antiserum dilution.

*Effect of Added Serum Protein on the Salt Optimum.*

In the light of the observations of Northrop and de Kruif (1922 a, b) an experiment to determine the effect of protein on the salt optimum was carried out. The technique used was the same as that described above except that the antiserum was diluted in distilled water containing one part in 87·5 of dialysed normal rabbit serum. With the wide range of antigen-antibody ratios set up in this experiment the salt optimum remained constant at M/14.

*Effect of Various Salt Concentrations on the End-point of Agglutination.*

Duncan *(loc. cit.)* found that the end-point of agglutination with any antiserum was determined in part by the concentration of salt in the system. Similar results were found with the brucellar system under examination. The results, given in Table 2, show that with decreasing salt concentration there is a falling-off in the end titre of the serum producing agglutination. It will be observed that the prozone at first shortens with decreasing salt and then lengthens, so that the only tube which presumably would show agglutination at a salt concentration somewhere between M/56 and M/112 would be that containing the constant antigen optimal ratio of organisms to antibody.

TABLE 2.

*Results of Experiment to Determine Effect of Various Concentrations of Sodium Chloride on the End-titre of Antiserum.*

| Antiserum Dilution. | Salt Concentration. 2M/7 | M/7 | M/14 | M/28 | M/56 | M/112 |
|---|---|---|---|---|---|---|
| 1/80 | f. tr. | tr. | + | + | — | — |
| 1/160 | + | + | + | + | tr. | — |
| 1/320 | + | + | + | + | + | — |
| 1/640 | ++ | ++ | ++ | ++ | ++ | — |
| 1/1280 | + | + | + | + | tr. | — |
| 1/2560 | + | tr. | f. tr. | — | — | — |
| 1/5120 | f. tr. | — | — | — | — | — |

+, ±, tr. (trace), f. tr. (fine trace), v. f. tr. (very fine trace) indicate degrees of agglutination.
— indicates no agglutination.

*Effect of Salt on Antiserum.*

Antiserum diluted 1/10 in distilled water was added to equal volumes of salt solution at double the required concentration. The mixtures were allowed to stand for half an hour at room temperature, when the salt concentrations of all mixtures were adjusted to M/14 by the addition of distilled water. A series of two-fold dilutions was then made from each and set up against a bacterial suspension of $1000 \times 10^6$ organisms per ml. The results are given in Table 3. In addition to the appearance of double ones in two cases, the results show that in concentrations above M/14 sodium chloride the zone of agglutination widens and there is an indication that the titre, as determined by the end-point of agglutination, is slightly lowered.

TABLE 3.

*Results of Experiment to Determine Effect of Sodium Chloride on Antiserum.*

| Concentration of salt to which antiserum exposed. | Antiserum dilution. 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | 1/5120 | 1/10240 | 1/20480 |
|---|---|---|---|---|---|---|---|---|
| 2M/7 | — | — | ± | + | + | — | — | — |
| M/7 | — | f. tr. | tr. | + | + | — | f. tr. | — |
| M/14 | — | — | v. f. tr. | + | + | v. f. tr. | — | — |
| — | f. tr. | — | f. tr. | + | + | f. tr. | — | — |

The signs used have same meaning as in Table 2.

## DISCUSSION.

In an agglutination reaction several factors operate.

Firstly, there is the combination of antibody with the antigenic groupings on the surface of the organisms, the amount of antibody combining with such groupings being determined by the ratio of antigen to antibody present in the mixture (Eisenberg and Volk, 1902). For any constant number of organisms there is a given amount of antibody which will optimally sensitize these organisms and provide the maximum cohesive force (*vide* Duncan, 1934). This amount of antibody corresponds with that present in the constant antigen optimal ratio (Ramon, 1922, Duncan, *loc. cit.*).

Secondly, there is the formation of aggregates due (a) to the reduction by the electrolyte of the electric charge upon the surface of the organism (Northrop and de Kruif, 1922) and (b) the cohesion of the sensitized organisms specifically (Marrack, 1934) and perhaps by reason of the attraction existing between two masses in close association.

Thirdly, there is the effect of the electrolyte on this cohesive force. Northrop and de Kruif have shown that in addition to decreasing the difference of potential between the liquid and the organism, the electrolyte also decreases the cohesive force between the organisms.

Whether the sensitized organisms will become agglutinated, therefore, in the presence of electrolyte will depend upon the nature of the resultant of these two effects. The effect of the serum protein in inhibiting, at least in part, the action of the salt in decreasing the cohesive force between organisms must not be overlooked (*vide* Northrop and de Kruif, *loc. cit.*). Such action has been demonstrated in the present work.

Fourthly, with decreasing concentrations of organisms, optimally sensitized or otherwise, per unit volume the number of contacts between organisms in unit time decreases markedly. This effect may be counteracted in part by increasing the amount of electrolyte present, thereby still further decreasing the charge on the surface of the organisms. The degree to which the concentration of electrolyte may be increased is limited, however, by its effect upon the cohesive force between the organisms.

The behaviour of any agglutinating mixture, therefore, should be capable of interpretation in the light of these facts.

A perusal of the results given in Table 1 suggest, because of the wide distribution of the salt optima for different suspension at a 1 : 700 dilution of antiserum, that the results obtained at this particular antiserum concentration constitute a suitable starting-point for the discussion.

Let us consider, firstly, the behaviour of the constant antigen optimal mixture ($1000 \times 10^6$ organisms to 1/700th ml. antiserum).

As shown by Duncan (1937) this mixture represents the one in which the cohesive forces are at a maximum and presumably, therefore, a minimum reduction in surface charge would lead to agglutination. Of the salt concentrations used in these experiments any one greater than M/112 proved capable of bringing about this necessary reduction. The greater the amount of salt present, however, the greater would be its action in lessening the cohesive force, and one finds therefore that the minimal salt concentration capable of producing agglutination is also the optimal concentration for this ratio of antigen to antibody.

With a number of organisms in excess of that present in the constant antigen optimum, the cohesive force is less as the result of imperfect sensitization. Such organisms require for optimal precipitation a greater reduction in their surface charge, which can be brought about by increasing the salt content; the effect of the salt in lessening the cohesive forces is then counteracted by the increase in the number of contacts per unit time.

With numbers of organisms less than that present in the constant antigen optimum the cohesive force is also less and more salt is required to produce optimal agglutination. In these cases, however, the number of possible contacts per unit time is less than in the constant antigen optimal mixture; this, together with a lessened cohesive force due to the over-sensitization and the action of salt, leads to a considerable slowing-up of the second stage of agglutination. In mixtures

containing one-quarter of the number of organisms present in the constant antigen optimum, visible particles appear only after 24–36 hours' incubation at 52° C.

With increasing antiserum concentrations the salt optimum of the constant antigen mixture moves to a higher concentration of the electrolyte. The cohesive force of the sensitized organisms remains constant but, because of the action of serum in inhibiting the effect of salt on this cohesive force, the balance between the two effects of the electrolyte is reached at a higher salt concentration.

With numbers of organisms in excess of that present in the constant antigen optimum, similar conditions exist as described above for an antiserum dilution of 1 : 700 except that in the presence of more serum the action of the salt on the cohesive force is less. At serum concentrations of 1/350th ml. or greater the inhibiting action of the salt on this cohesive force is not manifested and the optimum salt concentration is that at which a balance is reached between the degree of sensitization, the degree of reduction of surface charge and the number of contacts per unit time; this concentration is M/14. With numbers of organisms less than that present in the constant antigen optimum, most rapid agglutination takes place at much higher concentration of electrolyte, the limit being determined by the concentration of serum present. The greater the amount of serum present the greater the salt concentration required to give maximum reduction of surface charge without exerting an inhibitory action on the cohesive force.

At a serum dilution of 1/1400 it will be noted that the relative positions of the salt optima of the various ratios of antigen to antibody remain the same as at the 1/700 serum dilution, but that there is a general shift of the optima towards higher salt concentrations. Under the conditions of the experiments no salt optimum was obtained at a 1/2800 dilution of antiserum for any of the suspensions. The results obtained, however, indicate that such optima lie at very high salt concentrations, and the slope of the curves support this belief. (Fig. 1.)

It is interesting to note the relationship of the salt optima of the two antigen-antibody optimal mixtures. The constant antibody optimal mixture has its salt optimum at M/14 up to a serum dilution of 1/700; it then moves gradually to higher salt concentrations, owing no doubt to the falling-off in the number of organisms per unit volume. The salt optimum of the constant antigen optimal mixtures remains constant at M/14 only in serum dilutions lower than 1/350. Above this dilution it falls to M/56 at 1/700 serum and then gradually moves up again. Decreasing numbers of organisms per unit volume probably account for this subsequent rise. The factors responsible for the fall in the salt optimum concentration have already been discussed. The evidence available indicates also that for light suspensions ($2{\cdot}5 \times 10^9$ approximately or less) there are, at any salt concentration within a limited range, two serum concentrations at which the rate of particulation could not be increased either by increasing or by decreasing the concentration of electrolyte (see Table 1 and Fig. 1). One of these concentrations may

coincide with an *antigen-antibody* optimum. There is no evidence to indicate that these serum concentrations produce zones or that they constitute optima. In all cases the true antigen-antibody optimal mixture particulates first and the zone

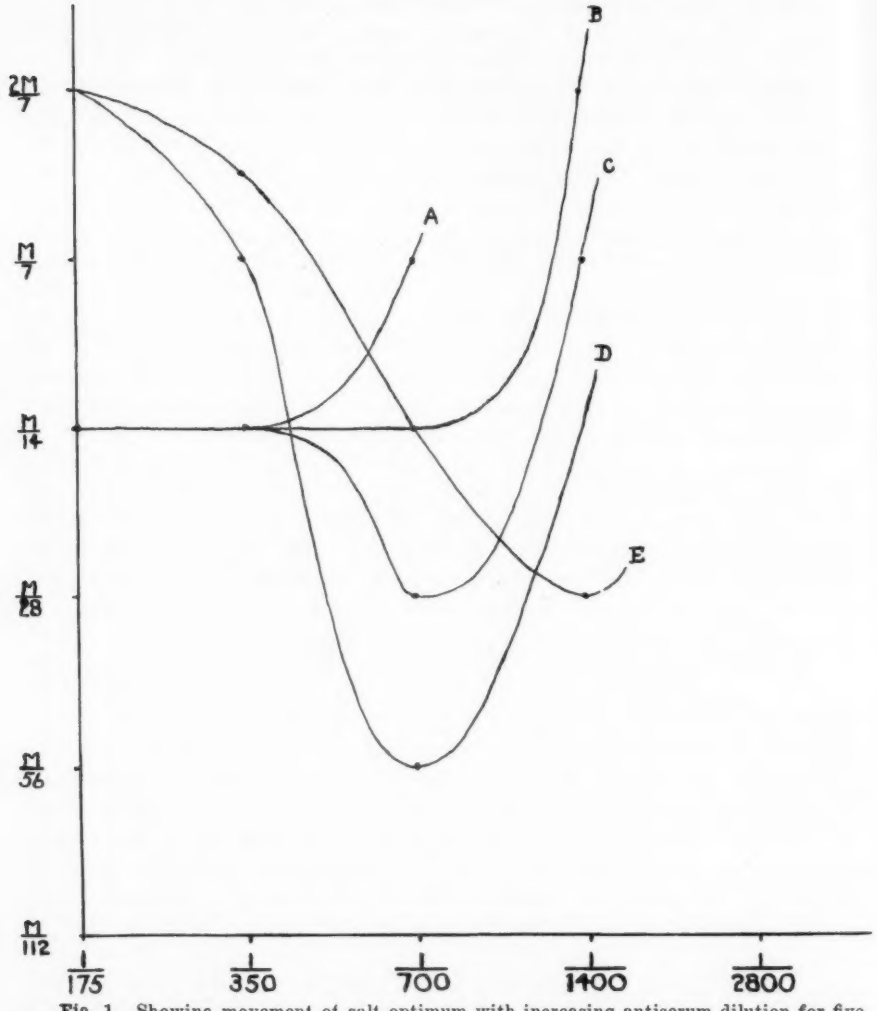



**Fig 1** Showing movement of salt optimum with increasing antiserum dilution for five different suspensions of *Br. abortus*. A, 5·714 × $10^8$ organisms per ml.; B, 2·857 × $10^9$ organisms per ml.; C, 2·000 × $10^9$ organisms per ml.; D, 1·000 × $10^9$ organisms per ml.; E, 0·500 × $10^9$ organisms per ml.

of agglutination extends progressively to the antigen excess and antibody excess side. The relation of these critical salt concentrations to the antigen and antibody optima will be discussed in a subsequent paper.

## SUMMARY.

1. Using a smooth strain of *Brucella abortus* and a corresponding antiserum obtained from an infected cow it was found that the *antigen* and *antibody* optimal ratios remained constant for all salt concentrations between 2M/7 and M/56. Concentrations of salt above 2/M7 were not used and those below M/56 failed to agglutinate the sensitized organisms.

2. The optimal salt concentration for any agglutinating system depends upon (a) the ratio of antigen to antibody; (b) the concentration of organisms per unit volume of mixture; (c) the effect of the salt on the cohesive force between the organisms.

## REFERENCES.

Bier, O. G. (1931): C.R. Soc. Biol., 108, p. 511.
Duncan, J. T. (1934): Brit. J. exp. Path., 15, p. 23.
Duncan, J. T. (1937): *Ibid.*, 18, p. 108.
Eisenberg, P. and Volk, R. (1902): Z. Hyg. Infektkr., 40, p. 155.
Holth, H. (1911): Z. Infektkr. Haustiere, 10, p. 207.
Marrack, J. R. (1934): Sp. Rep. Ser. M.R.C., No. 194.
Northrop, J. H. and Kruif, P. H. de (1922a): J. gen. Physiol., 4, p. 639.
Northrop, J. H. and Kruif, P. H. de (1922b): *Ibid.*, 4, p. 655.
Pandit, S. R. and Wilson, G. S. (1932): J. Hyg. Camb., 32, p. 45.
Ramon, G. (1922): C.R. Soc. Biol., 86, p. 711.

# A CASE OF RELAPSE IN EXPERIMENTAL POLIOMYELITIS

by

W. G. HEASLIP[1]

(From the Institute of Medical and Veterinary Science, Adelaide, South Australia).

*(Submitted for publication 5th September, 1938.)*

In his handbook Römer (1913) described a relapse in a monkey apparently recovered from an attack of poliomyelitis. This appears to be the only unequivocal case on record, although Levaditi and Stanesco, Leiner and v. Wiesner [cited in "Poliomyelitis", 1932 (a)] and Harmon *et al.* (1931) describe possible cases. Relapses in human beings appear to be not uncommon [see "Poliomyelitis", 1932 (b)]. In the recent epidemic in South Australia there were twenty-one deaths. Five of these occurred during convalescence, although apparently they were not true relapses. It is, therefore, of interest that a monkey, inoculated with this strain of virus, suffered a fatal relapse, and the event is here recorded.

M.735. 19/5/38. Inoculated intracerebrally with 1 c.c. of a mixture of 20 per cent. virus-suspension and human serum.

30/5/38. Paresis of the muscles of both legs.

31/5/38. Partial paralysis of both legs.

1 to 20/6/38. Recovery with residual paresis of both legs.

21/6/38. Animal appeared ill. Anorexia, coarse tremor and paralysis of right arm present. By evening of the same day the animal was prostrate.

22/6/38. Temperature subnormal. On stimulation the left arm and right leg moved feebly; the right arm and left leg were practically immobile. There was also paralysis of the muscles of the neck and some of the muscles of the face. The animal was killed with chloroform.

Macroscopically, the only abnormalities were areas of intense congestion at the hila of the lungs. The cord and brain were removed and material from both taken for section. The remainder was stored in glycerin in the ice-box.

The microscopic findings were as follows: In the cortex and at the levels examined from the cervical and lumbar enlargements of the spinal cord, tissue and perivascular infiltration was far more intense than is the case in the acute stages, and indicated the duration of the morbid process. Many of the anterior-horn nerve cells were partly degenerated, but could not be distinguished from those in a partially paralysed case surviving for several weeks without relapse. As a possible indication of acute changes which might account for the extension of paralysis,

[1] Working with a personal and expenses grant from the National Health and Medical Research Council.

polymorphonuclear leucocytes were present in considerable numbers in foci in the tissue and lying separately in vacuoles in the nerve cells. In the brain stem, on the other hand, the changes clearly provided an anatomical basis for recent extension of the paralysis, in the shape of acute destruction of nerve cells with neuronophagia obviously developed within a day or two preceding death. There was infiltration of the tissue with polymorphonuclear leucocytes as well as microglial cells.

To demonstrate the presence of the virus of poliomyelitis (and none other) in the cord the following experiments were carried out:

Three monkeys were inoculated; one M.731 had recovered from an attack of poliomyelitis, while M.769 and M.770 had not been used previously. The stored portions of the cord from M.735 were used to make a 10 p.c. suspension in saline. Part of this was mixed with an equal volume of known immune serum, incubated for one hour at 37° C. and then stood in the ice-chest overnight.

23/6/38. M.731 and M.769 inoculated with 1 c.c. of the suspension intracerebrally and 2 c.c. intramuscularly.

24/6/38. M.770 inoculated with 1 c.c. of the suspension-immune serum mixture intracerebrally and 4 c.c. intramuscularly.

M.769 developed the following signs:

3/7/38. Temperature 104·2°F. Irritable.

4/7/38. Temperature 105·0°F. Very irritable. Hypersensitive.

5/7/38. Temperature 104·2°F. Paresis of leg muscles.

6/7/38. Temperature normal. Legs weak.

7/7/38. Temperature normal. Legs paralysed.

The following day the animal was killed with chloroform and the lumbar enlargement of the spinal cord removed for section. The sections showed typical lesions of acute poliomyelitis.

M.731 and M.770 were observed for four weeks but showed no signs of infection.

To demonstrate that M.770 was susceptible and had been protected by the immune serum, it was inoculated with a mixture of 20 p.c. of virus-suspension and normal rabbit serum on 2/8/38. Typical poliomyelitis developed in six days and the animal was prostrate on 10/8/38.

## SUMMARY.

A case of relapse in experimental poliomyelitis is recorded. The presence of acute lesions in the brain-stem and of the virus of poliomyelitis in the cord of the animal were demonstrated to establish the diagnosis.

## REFERENCES.

Harmon, P. H., Shaughnessy, H. J. and Gordon, F. B. (1931): J. prev. Med., 5, p. 139.

Poliomyelitis: International Commission for the Study of Infantile Paralysis, Baltimore (1932): (a) pp. 93 and 112; (b) p. 187.

Römer, P. H. (1911): Die epidemische Kinderlähmung, Berlin, p. 80; (1913) English translation, London, p. 59.

# VITAMIN C NUTRITION AND SUSCEPTIBILITY TO DIPHTHERIA AS POSSIBLE FACTORS IN THE EPIDEMIOLOGY OF POLIOMYELITIS

by

W. G. HEASLIP[1]

(From the Institute of Medical and Veterinary Science, Adelaide, South Australia).

*(Submitted for publication 1st September, 1938.)*

Towards the end of 1937 an epidemic of poliomyelitis began in South Australia. A number of the earlier cases occurred in a city area where immunization against diphtheria had been carried out in the previous year and earlier in the same year, and Dwyer (personal communication) observed that the majority were in non-immunized children. Immunization had been carried out without previous Schick-testing. In 1937 also, Jungeblut reported that vitamin C had a therapeutic effect when injected subcutaneously into monkeys inoculated with the virus of poliomyelitis. A review of the literature on susceptibility to diphtheria and to poliomyelitis showed in each case a correlation with the results of certain experimental work with vitamin C, and suggested the investigation detailed below.

## LITERATURE.

### *Susceptibility to Diphtheria and Poliomyelitis.*

In 1917 Zingher reported that a disproportionately large percentage of cases of poliomyelitis gave a positive reaction to the Schick test. Of 954 cases between 1 and 4 years of age 81 per cent. were positive, whereas normal controls varied from 30 to 40 per cent. These figures are in accord with the observation of Baginsky (quoted by Jungeblut (1936)), that is is difficult to keep cases of poliomyelitis in hospital wards free from diphtheria. That there is nothing specific in the implied relationship is suggested by the fact that Zingher (1917) found that cases of scarlet fever also gave an unduly large percentage of positive reactions to the Schick test. This has been supported by other observations (Armstrong and Harrison, 1933; Jungeblut *et al.*, 1934).

[1] Working with a personal and expenses grant from the National Health and Medical Research Council.

Nevertheless, investigators, particularly Jungeblut and his co-workers, have produced sufficient evidence to make it probable that there is a basic factor underlying susceptibility to the two diseases. These observations include the following:

(a) Jungeblut (1934) has reported:

1. That certain samples of anti-diphtheritic horse serum are capable of neutralizing the virus of poliomyelitis.
2. That active immunization of rhesus monkeys against diphtheria protected some of them from paralysis when subsequently infected with poliomyelitis.
3. That a batch of normal rhesus monkeys gave a big majority of positive reactions to the Schick test, whereas a batch of similar monkeys convalescent from poliomyelitis gave a majority of negative reactions, though they were not immune to infection with diphtheria.

(b) Doull (1930) has shown that there is with age an increasing resistance to diphtheria apart from the specific resistance arising from contact with the disease. That the same is true of poliomyelitis is not absolutely certain, but the epidemiology of the disease makes it not improbable ("Poliomyelitis", 1932, p. 448). This belief is supported by arguments, in favour of the theory of physiological maturation, to explain the origin of virucidal substance in the serum (Jungeblut *et al.*, 1932, 1934).

(c) Jungeblut and Zwemer (1935) reported that *in vitro* vitamin C was capable of neutralizing diphtheria toxin. Jungeblut (1935) subsequently reported that this substance also neutralized the virus of poliomyelitis, and that both toxin and virus could be neutralized with adrenalin and with cortin. Holden and Resnick (1936), Sigal and King (1937) and Pakter and Schick (1938) attributed this effect of vitamin C to the acidity of the solution, but it was later shown that within certain limits neutralization is effected at a pH that is not inimical to the virus.

(d) Greenwald and Harde (1935) and Jungeblut and Zwemer (1935) have reported that vitamin C may protect guinea-pigs against the action of diphtheria toxin, although no such effect could be obtained in children (Pakter and Schick, 1938). Jungeblut (1937) has reported that 50 per cent. of monkeys treated with optimal doses of natural vitamin C survive infection with poliomyelitis.

### *Vitamin C and Susceptibility.*

These observations on the effect of vitamin C not only support the suggestion of a basic factor underlying susceptibility to the two diseases, but apparently indicate that this susceptibility is not necessarily absolute. It is probable that this basic factor is an inherent inability to react effectively to attempted or actual invasion by pathogenic organisms, or to the action of toxins which may enter or arise within the body. That such constitutional defect may exist is well known to clinicians and has been demonstrated in the case of diphtheria toxin by Hirszfeld *et al.* (1924)[2]. In the case of poliomyelitis we are still uncertain of the nature of the immunity that so generally prevails, though Fairbrother and Hurst (1930) have demonstrated the pathways by which the virus spreads through the nervous system. Their observations furnish an explanation of why immune-serum therapy has been unsuccessful. Since the virus may be intracellular from the time it invades the terminal cells of the olfactory system until the end of the disease, except when crossing the synaptic junctions between cells, it cannot be neutralized by antibodies in the serum. It is further protected by the functional barrier between the circulating blood and the

[2] Up to the time of submitting this paper for publication, eleven convalescing cases of poliomyelitis have been readmitted to the Metropolitan Infectious Diseases Hospital suffering from diphtheria. All of these had been immunized after admission to the Adelaide Children's Hospital for treatment of residual paralysis. Only three have contracted scarlet fever.

central nervous system, as is quite evident in certain other virus diseases where circulating antibodies develop early but do not stay the invasion of the central nervous system (Hurst, 1936).

The logical deduction from these observations is that the most hopeful method of protecting against the disease is to increase the ability of the patient to react effectively to attempted or actual invasion by the virus. That this is a possibility is apparent from the effect of vitamin C therapy in animals. In recent years much work has been done on the relation of vitamin C to infections, in which various workers have shown that there is a greatly increased utilization of the vitamin (Abassy *et al.*, 1935; Harris *et al.*, 1936; Bullowa *et al.*, 1936; Faulkner and Taylor, 1937). Likewise it has been reported (Lyman and King, 1936; Torrance, 1937; Jungeblut and Feiner, 1937; Harris *et al.*, 1937) that animals dying from the effects of diphtheria toxin or from poliomyelitis show a reduction of vitamin C in the tissues, indicating greater utilization at such a time. On the other hand, Jungeblut and Feiner (1937) found that monkeys given large doses of vitamin C before inoculation with poliomyelitis not only succumbed to the disease, but at the height of paralysis had tissue-storage levels of this substance equal to or above normal. It thus seems that the reported protection is not simply the result of supplying an increased demand for vitamin C. This is also suggested by the fact that the range of effective subcutaneous dosage was small and the optimal dose was very small, opposing the possibility that the effects of vitamin C administration are due to overcoming a preceding deficiency. Obviously further work is needed to elucidate the apparent contradictions herein.

### *Vitamin C and Prophylaxis.*

As stated above, Jungeblut (1937) was completely unsuccessful in his attempt to protect monkeys against the disease by oral and subcutaneous administration of vitamin C before inoculation with the virus of poliomyelitis. It has been argued ("Poliomyelitis", 1932, p. 427) that nutrition in general has no bearing on the epidemiology of the disease in man. However this does not necessarily mean that vitamin nutrition has no bearing. It has frequently been observed that a vitamin deficiency may predispose to infection (Medical Research Council, Special Report No. 167, 1932, pp. 28 and 193; Giroud, 1936; Aykroyd, 1936). Clausen (1934) emphasized that a deficient diet does not seem to increase susceptibility to infection but definitely does decrease resistance, presumably by local action. It is significant in this connection that there is an increasing recognition of abortive cases of poliomyelitis. During the present investigation McCormick (1938) suggested that the rising age-incidence of poliomyelitis may be the result of greater attention to the nutritional needs of infants and children, and particularly to the supply of vitamin B.

That vitamin C plays an important part in toxaemias and infections, has been observed in animal experiments (Clausen, 1934; Harde and Benjamin, 1935; Giroud, 1936; Harris *et al.*, 1937) and with human subjects. During the course of the diseases investigated, which include tuberculosis (Faulkner and Taylor, 1937; Abassy, Harris and Ellman, 1937), influenza and coryza (Abassy *et al.*, 1935, 1937), pneumonia (Harde *et al.*, 1935; Bullowa *et al.*, 1936), osteomyelitis (Abassy *et al.*, 1937), rheumatoid arthritis (Abassy *et al.*, 1937), pertussis (Ormerod *et al.*, 1937), Addison's disease (Wilkinson and Ashford, 1936), poliomyelitis, scarlet fever and diphtheria (this paper), there is a lowered excretion of vitamin C.

As indicated above there is considerable doubt as to the relative importance of the two sequences, viz., low level of nutrition decreasing resistance to disease, and disease resulting in increased utilization of vitamin C. We know that both sequences occur, and certain observers could not state whether nutrition was low before infection or had been lowered by infection. Faulkner and Taylor (1937) observed that an original deficiency was practically unaffected by infection. On the other hand there is abundant evidence to show that infection causes a greatly increased utilization of vitamin C. Here again controlled investigations are needed to demonstrate the actual relation of vitamin C nutrition to prophylaxis in disease.

## PRESENT INVESTIGATIONS.

In an endeavour to obtain further information concerning these questions the following investigations were made:

1. The first was an attempt to confirm Zingher's findings and to amplify them by correlating the result of Schick-testing with other observations. The findings are given in Tables 1 and 2.

2. The second was an estimation of the urinary excretion of vitamin C from cases of poliomyelitis and others, to establish if possible the surmise that during these acute infections there is a reduced output. The findings are set out in Tables 3 and 7.

3. The third was an attempt to determine the state of vitamin nutrition of South Australian urban children in general, and in particular of children who had been in contact with cases of poliomyelitis but had not contracted the disease. Also, to determine the relation, if any, between the state of vitamin nutrition and the age-incidence in this epidemic. This is shown in graph form in Fig. 1. The total incidence is shown in Table 6.

4. The fourth was to correlate the results of 1 and 2 with the type of case investigated. For this purpose the cases were arbitrarily classified as severe, moderate or mild by an independent observer[3]. The figures are set out in Tables 2, 4 and 5.

## METHODS.

The Schick-testing and the reading of results were performed according to the instructions issued by the Commonwealth Serum Laboratories whence the supply of toxin was obtained. In each case a control injection was also done.

The group of children comprising the controls was tested within the period of the epidemic by the Officer of Health for Hindmarsh, and by his kind permission the reading of results was checked to obtain uniformity in both groups.

Vitamin C excretion was estimated by the method of Harris and Ray (1935) with a modification made necessary by the difficulty of collecting the specimens. Urine was collected as 24-hour specimens and the total volumes recorded. To each quantity of urine, as soon as it was passed and measured, 10 per cent. of glacial acetic acid was added as a preservative. Six ounces of each 24-hour specimen were saturated at the laboratory with sulphuretted hydrogen. After 24 hours in the dark, excess sulphuretted hydrogen was removed by briskly bubbling carbon dioxide through the specimen which was then titrated as soon as possible with 2 : 6 dichlorophenolindophenol. As determined by preliminary test the result obtained represented approximately 75 per cent. of the actual amount of vitamin C excreted in the 24 hours. Since we required only comparative estimations, the method proved quite satisfactory. Three 24-hour specimens were obtained from each patient. As soon as the last portion of the first and second 24-hour specimens was collected the patient was given in water a dose of ascorbic acid equal to 5 mg. per lb. body-weight. Patients were deprived of fresh fruit, including tomatoes and fruit drinks, from the time they were first seen until the test was completed. The control groups were tested in the same way.

---

[3] We are indebted to Dr. A. Finger, Superintendent of the Metropolitan Diseases Hospital, Northfield, for this classification.

In the third investigation the figures obtained in the second were used. In addition the figures relating to age and incidence in the epidemics in this and neighbouring States were obtained by courtesy of the Health Officers concerned. The age-incidence graphs depict the percentage of cases in each 5-year age group.

## DISCUSSION OF RESULTS.

*Schick Tests.*

Jungeblut (1936) cites an attempt in 1933 to confirm Zingher's findings. The result showed only 40 p.c. of positive reactions. This low figure may have resulted from immunization carried on between 1916 and 1933 in New York, as suggested by the finding of Henry and Johnson (1934), who reported in the latter year from Philadelphia that of 304 cases of poliomyelitis 51 per cent. had been immunized against diphtheria. The figures given in Table 1 as a result of the present investigation are not large enough to have statistical significance alone, but they serve to confirm Zingher's findings, and emphasize the persisting susceptibility to diphtheria in these cases.

TABLE 1.

*Showing the Results of Schick-Testing Cases of Poliomyelitis and Normal Children.*

| | | | | | Non-immunized Patients. | | |
|---|---|---|---|---|---|---|---|
| | | Number tested. | Number positive. | Percentage positive. | Number tested. | Number positive. | Percentage positive. |
| Cases of Poliomyelitis | Under 6 years | 72 | 57 | 79·2 | 67 | 56 | 83·6 |
| | 6–12 years | 83 | 53 | 63·9 | 65 | 48 | 73·8 |
| | 12 years and over | 83 | 47 | 56·6 | 76 | 45 | 59·2 |
| | Total | 238 | 157 | 66·0 | 208 | 149 | 71·6 |
| | Under 12 years | 155 | 110 | 71·0 | 132 | 104 | 78·8 |
| | Under 4 years | 48 | 38 | 79·2 | 45 | 38 | 84·4 |
| Controls (under 12 years) | | | | | 207 | 118 | 57·0 |

In the table both the total cases and the non-immunized cases are set out. This has been done firstly, to make a true comparison with the control group which contained no immunized cases; and secondly, because the figures relating to the immunized group are interesting.

The control group contained no immunized children as it was the non-immunized portion of a larger group. It was not in any sense a selected group, however, as the immunized children had not been Schick-tested prior to immunization.

Comparing the non-immunized cases of poliomyelitis with the controls, we find that the age-group of 12 years and over gives substantially the same percentage of positive reactions as the control group. This is possibly the most extraordinary result obtained, as this age-group contains 42 p.c. of the cases and their ages range up to 60 years. Thirty per cent. of them are 21 years or over. Admitting that

some of these are rural dwellers, the figure is still very high in a State where diphtheria is moderately prevalent. The same may be said of the whole group, especially when one considers that less than a third of the cases are under six years of age. Of the 238 cases tested 21 had been artificially immunized and nine had had diphtheria. Of these 30 cases, eight (26·7 p.c.) gave a positive reaction to the Schick test. In a control group of 359 cases, of which four had had diphtheria and 355 had been artificially immunized, only two gave a positive reaction. One of these had received only 0·5 c.c. of anatoxin and the other only 1 c.c. One is tempted to suggest that the latter result is due to the fact that those who remained Schick-positive after immunization contracted poliomyelitis. These figures support the idea of a constitutional defect underlying susceptibility to the two diseases, and it is not surprising to learn that several of the cases of poliomyelitis have, within a few months, contracted diphtheria despite immunization.

Finally, as stated above, Dwyer reported that very few of these immunized cases contracted poliomyelitis. This contrasts sharply with the report of Henry and Johnson that 51 p.c. of their cases of poliomyelitis had been previously immunized. The explanation is probably that Dwyer's group was immunized without previous Schick test, that is to say many of them did not require it, whereas it is justifiable to assume that all of the latter group were Schick-positive prior to immunization and required it.

From these results, supported as they are by Zingher's observations on a much larger group, it is evident that there is a greater susceptibility to poliomyelitis in persons giving a positive Schick reaction than in those who give a negative. Conversely there is a larger percentage susceptible to diphtheria amongst those who contract poliomyelitis than in the normal population. Also there is evidence here that an underlying factor in this phenomenon is a constitutional defect.

TABLE 2.

*Showing the Relationship between the Reaction to the Schick Test and the Severity of the Attack of Poliomyelitis.*

| Type of Case. | Severe. | | Moderate. | | Mild. | | Total. | |
|---|---|---|---|---|---|---|---|---|
| | Number. | P.c. | Number. | P.c. | Number. | P.c. | Number. | P.c. |
| Schick Positive | 36 | 23·2 | 62 | 40·0 | 57 | 36·8 | 155 | 100 |
| Schick Negative | 27 | 33·8 | 20 | 25·0 | 33 | 41·2 | 80 | 100 |
| Total | 63 | 26·8 | 82 | 34·9 | 90 | 38·3 | 235 | 100 |

In Table 2 the relation between the reaction to the Schick test and the severity of the attack of poliomyelitis is shown. If the conclusions drawn above are correct we should expect to find a larger percentage of severe cases and a lower percentage of mild cases in the Schick-positive group than in the Schick-negative group. But although there is a slightly higher percentage of mild cases in the latter group there is a decidedly higher percentage of severe cases. An explanation of this apparent discrepancy is submitted below.

*Vitamin C Excretion.*

It was thought that the amount of vitamin C estimated from the first of the three 24-hour specimens would give an idea of the normal excretion rate of the patient. This conjecture, however, proved to be unsound. The amount of vitamin C in the last two specimens has been expressed as the percentage of the amount given by mouth, and indicates fairly accurately the state of vitamin C nutrition of the patient. The figures are set out in Tables 3 and 7.

TABLE 3.

*Showing Urinary Excretion of Vitamin C from Cases of Poliomyelitis and Others.*

| | Number tested. | Excretion of Ascorbic Acid. In mg. for 24 hours before treatment. Highest. | Lowest. | Mean. | Percentage of Acid administered. Highest. | Lowest. | Mean.* |
|---|---|---|---|---|---|---|---|
| Cases of poliomyelitis | 60 | 144 | 4 | 27·78 ± 26·89 | 69 | 5 | 19·9 |
| Contacts who remained healthy | 45 | 128 | 3 | 21·06 ± 19·86 | 108 | 8 | 44·3 |
| Adequately fed normal children | 11 | 26 | 13 | 20·63 ± 3·47 | 120 | 13 | 58·9 |
| Suspected cases of poliomyelitis with normal cerebro-spinal fluid | 4 | 17 | 6 | 11·25 ± 3·89 | 16 | 7 | 12·3 |
| Cases of scarlet fever | 8 | 17 | 5 | 10·0 ± 3·57 | 52 | 2 | 14·8 |
| Cases of diphtheria | 12 | 81 | 5 | 24·0 ± 22·93 | 85 | 4 | 28·6 |

* The calculation of the statistical mean and the probable error of this mean was not attempted because of paucity of data obtainable. The actual findings are shown in Table 7.

The highest and lowest levels have been included in the table because there was a great variation in the amounts excreted. This occurred in all the groups tested. It is possible that the high level of excretion in some cases was due to the unauthorized consumption of fruit or fruit drinks, but this cannot be safely assumed. For example the case which excreted 144 mg. before any ascorbic acid was administered was under strict control.

This case, amongst others, was tested over a period of 5 days, in an attempt to confirm the irregular findings obtained previously, with the following results:

| | |
|---|---|
| 1st 24 hours on test diet | 44 mg. of vitamin C excreted. |
| 2nd " " " " " | 144 " " " " " |
| 3rd " " 590 mg. ascorbic acid given | 58 " " " " " |
| 4th " " 590 " " " " | 154 " " " " " |
| 5th " " on test diet | 42 " " " " " |

These figures are given in full to demonstrate that great irregularity of excretion occurs and that even a three-day test may not give a reliable absolute result. The unreliability of the one-day test is obvious, as Harde *et al.* (1935) have previously pointed out. The above was not an isolated case and irregularity was not confined to cases of poliomyelitis; on the other hand the degree of variation in the figures is not very significant when the amount of the test dose is considered.

Under these circumstances it was thought better to give the actual figures of the tests without advancing any definite conclusions, though the irregularities would hardly account for the disparity between the mean percentage excretion of the cases of poliomyelitis and the normal controls (see Table 3). This is borne out by the fact that the two control groups of normal children give the expected comparative results. It is not clear at present whether the mean results of the first-day excretion are significant or not. It is noticeable that the deviation from the mean is least in the adequately fed children and in those groups with very low excretion. Irregularity of excretion may account for the cases of poliomyelitis giving the highest figure. On the other hand this would necessitate a high degree of coincidence, more particularly as the two normal groups are practically the same. In this connection it is of interest to note that Torrance (1937) gave guinea-pigs sublethal doses of diphtheria toxin and found subsequently first a rise of vitamin C content in the adrenals and then a considerable fall. Be this as it may, the mean percentage excretion figures suggest that there is a relationship between poliomyelitis and a lowered vitamin C nutrition.

*Relation of Vitamin C Excretion to Type of Case.*

Figures showing the relation of the urinary excretion of vitamin C to the severity of the attack of poliomyelitis are included in Tables 4 and 5. They show that the excretion in the severe cases is lower than in the moderate and mild cases. Two factors account for the lack of difference between the moderate and mild cases. One is the low excretion in the Schick-negative cases, and the other is the low excretion in the rural as compared with the urban cases. These phenomena are discussed below. Out of the 23 moderate cases only four were Schick-negative; only eight were from rural areas; and the four Schick-negative cases were all from urban areas. All these facts tend to produce a comparative increase in the figures for the moderate group. On the other hand, seven of the 16 mild cases were Schick-negative. Of these, five were below average (21·9) and the other two were only 1·1 above it. When these factors are taken into account there is demonstrated a direct relationship between the level of nutrition and the severity of the attack, which recalls the report of Weisenburg (1917) of a case fatality of 67 out of 126 amongst artificially fed, and only seven out of 49 amongst breast-fed infants.

TABLE 4.

*Showing the Relation between the Reaction to the Schick Test and the Urinary Excretion of Vitamin C: and the Relation of both to the Severity of the Attack of Poliomyelitis.*

| Type of Cases. | Numbers tested. | | | Average percentage of Ascorbic Acid. | | | Total. tested. | Average percentage excretion. |
|---|---|---|---|---|---|---|---|---|
| | Severe. | Moderate. | Mild. | Severe. | Moderate. | Mild. | | |
| Schick-positive | 14 | 19 | 9 | 19·0 | 21·8 | 26·2 | 42 | 21·8 |
| Schick-negative | 7 | 4 | 7 | 10·6 | 22·7 | 16·4 | 18 | 15·6 |
| Total | 21 | 23 | 16 | 16·2 | 22·0 | 21·9 | 60 | 19·9 |

A study of Table 4 reveals that in the Schick-positive group there is a relationship between the severity of the attack and the excretion of vitamin C. In the Schick-negative group this relationship is somewhat disturbed, although the excretion from the severe cases is lower than that from the mild cases. The relatively high figure for the moderate group is explained by the fact that all four cases are from urban areas. The relevance of this will appear later when a comparison is made between rural and urban groups. But it also reveals that the Schick-negative group has an unduly low excretion which the number of severe cases in the group does not explain. Previously we noted an unduly high proportion of severe attacks in this group. We have seen that a deficient vitamin C nutrition increases susceptibility to infection, and that the level of excretion is related to the severity of the attack in poliomyelitis. We may, therefore, be justified in assuming that in this group of cases the low excretion of vitamin C *preceded* the attack of poliomyelitis. This would account for the high proportion of severe attacks; the unduly low excretion of vitamin C; and the fact that Schick-negative cases became infected. These phenomena all remain unexplained unless this assumption is made.

One of the histories of nutrition that was obtained supports this hypothesis. Of the two girls in the family one was 9 and the other 3 years of age. Both were Schick-positive. The mother voluntarily stated that the younger would not drink milk nor eat fruit or green vegetables, whereas the elder partook freely of all. Despite the fact that more cases occurred in the epidemic in this State in the age-nine than in the age-three group, it was the younger who contracted the disease. She had a severe attack. Her first day excretion was 7 mg. and her percentage excretion was 10. Her sister's figures were 36 and 108 respectively. At the time of contact the latter had no neutralizing bodies in her serum but subsequently developed them.

TABLE 5.

*Showing the Relation between Vitamin C Excretion, and the Severity of the Attack of Poliomyelitis in Rural and Urban Groups of Cases.*

| Type of Case. | Numbers tested. Severe. | Moderate. | Mild. | Totals. | Mean percentage excretion of vitamin C. |
|---|---|---|---|---|---|
| Urban | 8 | 15 | 13 | 36 | 22·1 |
| Rural | 13 | 8 | 3 | 24 | 16·6 |
| Totals | 21 | 23 | 16 | 60 | 19·9 |

However, despite the foregoing, it is probable that in the majority of these cases the diminished excretion succeeded the onset of the disease, for the following reasons: (a) Controls from the same homes as the cases of poliomyelitis gave a much higher excretion than did the patients. (b) A dietary history was obtained from most of the families investigated, and in only two instances was the patient stated to vary from the contacts in the matter of food preference. (c) The control groups of cases of infection also showed a lowered excretion. The cases of suspected poliomyelitis are particularly significant. (d) There appeared to be a definite relationship between the severity of the case and the level of vitamin C nutrition.

(e) Other workers cited above have shown that the onset of an infection coincides with a lowering of excretion; that the latter is definitely related to the height of the fever; and that latent infection following an acute illness will keep the excretion at a low level.

These findings confirm the work of others, and the inference is that vitamin C nutrition in particular, as well as vitamin nutrition in general, may play an important part in the epidemiology of poliomyelitis.

*Vitamin Nutrition.*

At the present time in this State one would not expect to find many cases of vitamin deficiency. Clinical cases are rarely seen, and recently Turner (1938) has pointed out that the infant mortality here has dropped to a lower level than is found in the other Australian States. He attributes this to the fact that adequate supplies of milk have been made available to necessitous mothers and children. Fruit is not prohibitive in price, especially in the urban areas, and the absence of oranges in the summer months is made good by the abundance of tomatoes and other fruit. Consequently it is not surprising to find that the two groups of normal children tested for vitamin C excretion (*vide* Table 3) gave similar good results, although most of the contacts came from comparatively poor families.

An analysis of the individual figures of the contacts tested shows that only two had an excretion under 10 p.c. of the dose of ascorbic acid given. Both excreted 8 p.c. Four others had an excretion under 20 p.c. (13 p.c., 15 p.c., 16 p.c., and 19 p.c.). Remembering that these figures represent only 75 p.c., approximately, of the actual excretion—the loss was greatest with the lowest titres—it is clear that not more than a very small percentage of city children are deficient in vitamin C.

TABLE 6.

*Showing the Incidence of, and Case Fatality in, Poliomyelitis in Several Australian States.*

| State. | Population. | Number of cases. | Incidence per 1,000 of population. |
|---|---|---|---|
| Tasmania | 233,390 | 989 | 4·2 |
| Victoria | 1,859,487 | 2,059 | 1·1 |
| South Australia | 591,201 | 343 | 0·58 |

On the other hand, the state of nutrition in the rural areas is not so satisfactory. This would be expected from the different conditions and is revealed statistically in the nutrition survey carried out in 1936 by Clements (Fourth Report of the Advisory Council on Nutrition, 1937). Fourteen per cent. of inland children were found to be suffering from some nutritional deficiency, though no scurvy was seen. It is also reflected in the figures in Table 5. These show a lower excretion of vitamin C from the rural than from the urban cases. This

finding substantiates the conclusions drawn in the case of the Schick-negative group. In both instances there is a preponderance of severe cases associated with a low excretion, and in both the assumption that the vitamin deficiency *preceded* the attack explains all the associated facts. It is not feasible to explain the low excretion in the rural cases by the severity of the attack since this leaves unexplained, as in the Schick-negative group, the preponderance of severe cases in this group, and the fact that all the fatal cases tested came from rural areas. It is worth noting that of the six Schick-negative rural cases, two were fatal and three others severe. The average percentage excretion of these six was 10·2 (see Table 7).

TABLE 7.

*Ascorbic Acid Excreted.*

| Case. | Age. | Area. | Attack. | Schick test. | Before acid given. 1st day. | After acid given. 2nd day. | After acid given. 3rd day. | As percentage of amount given. |
|---|---|---|---|---|---|---|---|---|
| 9 | 17 | Urban | Severe | positive | 27 | 244 | 185 | 61 |
| 11 | 6 | " | " | " | 16 | 13 | 56 | 16 |
| 18 | 11 | " | " | " | 34 | 125 | 28 | 23 |
| 45 | 3 | " | " | " | 11 | 13 | 14 | 10 |
| 66 | 5 | " | " | " | 15 | 19 | 25 | 11 |
| 88 | 13 | " | " | " | 23 | 57 | 151 | 27 |
| 8 | 12 | " | " | negative | 5 | 5 | 128 | 21 |
| 33 | 10 | " | " | " | 7 | 9 | 48 | 10 |
| 67 | 5 | Rural | " | positive | 37 | 43 | 61 | 26 |
| 68 | 28 | " | " | " | 54 | 24 | 87 | 7 |
| 81 | 4 | " | " | " | 6 | 8 | 73 | 27 |
| 87 | 14 | " | " | " | 31 | 64 | 36 | 10 |
| 89 | 38 | " | " | " | 33 | 74 | 260 | 18 |
| 90 | 23 | " | " | " | 13 | 28 | 76 | 7 |
| 105 | 8 | " | " | " | 9 | 9 | 41 | 9 |
| 112 | 36 | " | " | " | 84 | 56 | 133 | 14 |
| 50 | 12 | " | " | negative | 19 | 35 | 53 | 12 |
| 84 | 54 | " | " | " | 19 | 79 | 61 | 10 |
| 86 | 11 | " | " | " | 14 | 19 | 14 | 5 |
| 91 | 16 | " | " | " | 48 | 58 | 69 | 11 |
| 98 | 16 | " | " | " | 19 | 36 | 35 | 5 |
| 1 | 6 | Urban | Moderate | positive | 6 | 11 | 124 | 34 |
| 4 | 7 | " | " | " | 105 | 50 | 47 | 20 |
| 12 | 8 | " | " | " | 11 | 21 | 35 | 12 |
| 20* | 4 | " | " | " | 6 | 47 | 101 | 43 |
| 69 | 7 | " | " | " | 10 | 17 | 30 | 8 |
| 76 | 4 | " | " | " | 4 | 21 | 9 | 8 |
| 79 | 4 | " | " | " | 8 | 16 | 75 | 25 |
| 85 | 19 | " | " | " | 26 | 113 | 164 | 23 |
| 92 | 14 | " | " | " | 7 | 20 | 87 | 13 |
| 96 | 19 | " | " | " | 11 | 23 | 215 | 34 |
| 108 | 4 | " | " | " | 10 | 20 | 23 | 12 |
| 21 | 8 | " | " | negative | 12 | 122 | 44 | 30 |
| 32 | 9 | " | " | " | 18 | 21 | 91 | 19 |
| 72 | 19 | " | " | " | 30 | 141 | 210 | 16 |
| 80 | 7 | " | " | " | 10 | 25 | 104 | 26 |
| 65 | 7 | Rural | " | positive | 19 | 51 | 93 | 29 |
| 71 | 14 | " | " | " | 144 | 58 | 154 | 18 |
| 77 | 9 | " | " | " | 20 | 78 | 121 | 33 |
| 82 | 5 | " | " | " | 16 | 25 | 74 | 25 |

TABLE 7 (continued).

| Case. | Age. | Area. | Attack. | Schick test. | Before acid given 1st day. | After acid given. 2nd day. | 3rd day. | As percentage of amount given. |
|---|---|---|---|---|---|---|---|---|
| 104 | 7 | Rural | Moderate | positive | 18 | 49 | 129 | 34 |
| 107 | 16 | " | " | " | 24 | 188 | 145 | 28 |
| 110 | 18 | " | " | " | 17 | 61 | 43 | 8 (1) |
| 111 | 5 | " | " | " | 5 | 10 | 19 | 7 |
| 30 | 15 | Urban | Mild | " | 113 | 118 | 245 | 35 |
| 34** | 15 | " | " | " | 18 | 192 | 501 | 69 |
| 47 | 15 | " | " | " | 18 | 46 | 61 | 11 |
| 51 | 8 | " | " | " | 19 | 45 | 22 | 13 |
| 52 | 15 | " | " | " | 22 | 184 | 253 | 36 |
| 70 | 15 | " | " | " | 11 | 67 | 179 | 20 |
| 109 | 5 | " | " | " | 7 | 23 | 31 | 14 |
| 26 | 16 | " | " | negative | 71 | 56 | 200 | 23 |
| 38 | 5 | " | " | " | 12 | 12 | 78 | 23 |
| 41 | 10 | " | " | " | 44 | 19 | 91 | 18 |
| 99 | 13 | " | " | " | 18 | 44 | 28 | 7 (2) |
| 100 | 9 | " | " | " | 28 | 23 | 64 | 12 |
| 106 | 8 | " | " | " | 33 | 24 | 55 | 14 |
| 78 | 7 | Rural | " | positive | 45 | 31 | 137 | 32 (3) |
| 97 | 7 | " | " | " | 12 | 11 | 18 | 6 |
| 64 | 8 | " | " | negative | 16 | 62 | 41 | 18 |

* A contact who developed poliomyelitis. Illness started the day after the test began. Paralysis apparent 10 days later.

** Test not started until 8th day after admission.

(1) 90 mg. ascorbic acid excreted on 4th day on normal test diet.

(2) 43 " " " " " " " " " " "

(3) 103 " " " " " " " " " " "

This association of severity of attack and higher incidence of poliomyelitis, with a positive reaction to the Schick test and/or a low level of vitamin C nutrition appears to be of considerable importance from the point of view of prophylaxis. In this connexion the consistently higher incidence of poliomyelitis in rural as compared with urban areas ("Poliomyelitis", 1932, p. 316) is highly suggestive. Urban dwellers may be expected to show a higher percentage of Schick-negative individuals and a lower proportion of cases of vitamin deficiency.

It appears probable that compulsory immunization against diphtheria and ensuring adequate vitamin nutrition in a community may be a justifiable and cheap form of insurance against the tragedy and expense of a high incidence of poliomyelitis. It is not suggested, however, that immunization against diphtheria is anything more than a stimulant; nor adequate vitamin nutrition other than one of the factors which render the organism as perfectly equipped as possible for defence against disease.

*Age-incidence and Vitamin Nutrition.*

For comparative purposes in a study such as this the large area of the separate Australian states makes it impossible to draw unqualified conclusions. For example the geographical factor must have a large influence on the incidence of polio-

myelitis. The figures in Table 7 show that Tasmania, which is furthest from the equator, has the highest incidence, and that Victoria, which is nearest Tasmania, has the next highest.

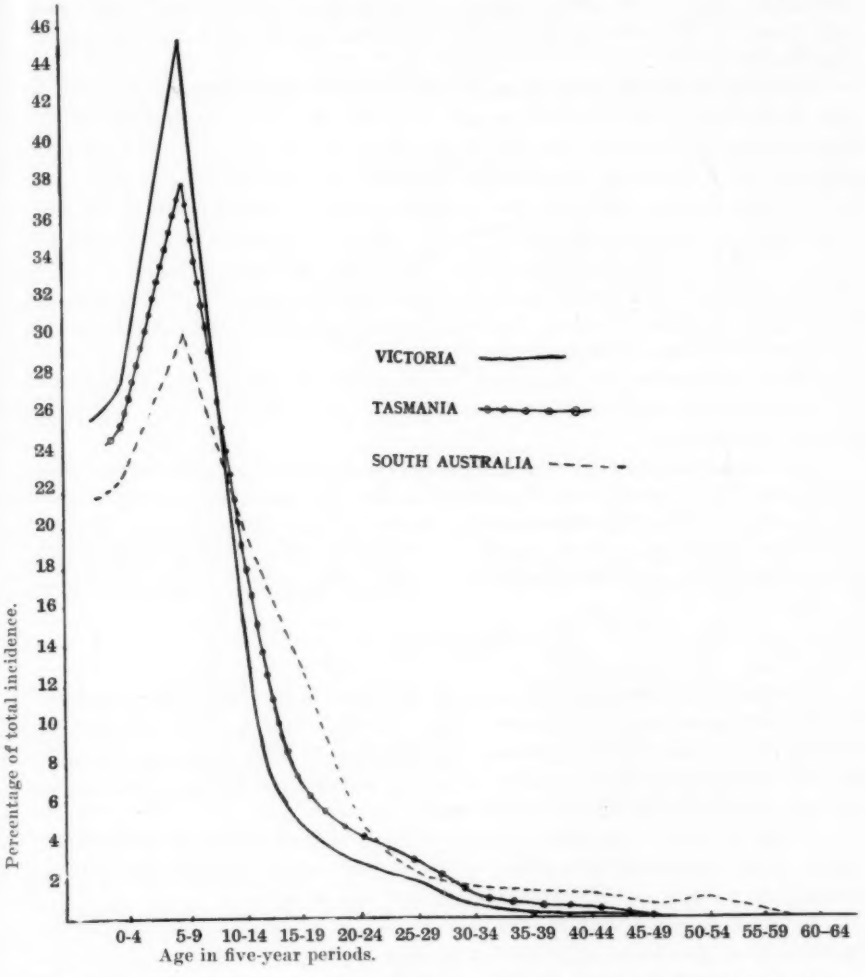



**Figure 1.** Showing the age-incidence in several Australian States in the last epidemic of poliomyelitis.

However, it is improbable that geographical situation would, of itself, affect the age-incidence. On the other hand it is probable that vitamin nutrition would do so. Weisenburg (1917) reported that there were 175 cases amongst infants under one year in the 1916 epidemic in Philadelphia. Of these infants only 49 were breast-fed. This observation is confirmed by the fact that in the epidemic here only six of the 343 cases of poliomyelitis were less than one year old, and none of these was breast-fed.

The Fourth Report of the Advisory Council on Nutrition (1937) gives the results of nutrition surveys in various parts of Australia. Unfortunately these have been carried out in certain sections of the community only. It follows that the different types of country investigated will modify the results. For example, conditions from a dietary point of view would be worse in north-eastern South Australia than in the Victorian mallee country. However, when these conditions are taken into account, it appears that there is a higher incidence of malnutrition in Victoria than in South Australia. From the conditions one would expect nutrition to be better in Tasmania than in Victoria, and from the infant mortality rate (Turner, 1938) worse than in South Australia.

These conclusions are confirmed by the graphs in Fig. 1 which show that the age-incidence was lowest in Victoria; highest in South Australia; and intermediate in Tasmania.

This suggests again that vitamin nutrition is associated with the epidemiology of poliomyelitis, and supports the suggestion that the phenomenon of increasing age-incidence may be due to greater attention to the nutritional needs of children.

Taken individually these observations would possibly be of doubtful value, but one feels that it is rather significant when they all point to the same conclusions.

## SUMMARY.

1. Cases of poliomyelitis and controls have been subjected to the Schick test and the findings of Zingher confirmed. From this and other observations it appears that there is a relationship between susceptibility to diphtheria and to poliomyelitis. This relationship probably (a) is not confined to these two diseases only; (b) has a constitutional defect underlying it.

2. The urinary excretion of vitamin C from various groups of infected and non-infected persons has been estimated and a relationship demonstrated between the infectious states and the level of vitamin C excretion.

3. Cases of poliomyelitis have been classified and a relationship shown between the severity of the disease and the level of the vitamin C nutrition.

4. An analysis of the results obtained has been made and the conclusion drawn that while the usual sequence of events is a decreased excretion of vitamin C following infection, it is probable that a low level of vitamin C nutrition predisposes to infection and severity of attack.

5. Observations and figures relating to vitamin nutrition (particularly vitamin C) and the incidence of poliomyelitis have been presented. It is probable that there is a relationship between nutrition and age-incidence in this disease.

## ACKNOWLEDGMENTS.

I wish to express my thanks to the Superintendent and staff of the Metropolitan Infectious Diseases Hospital, Northfield, for their helpful co-operation in part of this work.

## REFERENCES.

Abassy, M. A., Harris, L. J. and Ellman, P. (1937): Lancet, 2, p. 181.
Abassy, M. A., Harris, L. J. and Hill, N. G. (1937): *Ibid.*, 2, p. 177.
Abassy, M. A., Harris, L. J., Ray, S. N. and Marrack, J. R. (1935): *Ibid.*, 2, p. 1399.
Advisory Council on Nutrition, Fourth Report, Canberra, Australia, 1937.
Armstrong, C. and Harrison, W. T. (1933): U.S.A. Pub. Health Reports, 48, p. 597.
Aykroyd, W. R. (1936): Vitamins and other dietary essentials, 2nd Ed., London, p. 182.
Bullowa, J. G. M., Rothstein, I. A., Ratish, H. D. and Harde, E. (1936): Proc. Soc. exp. Biol. Med. 34, p. 1.
Clausen, S W. (1934): Physiol. Rev., 14, p. 309.
Doull, J. A. (1930): J. prev. Med., 4, p. 371.
Fairbrother, R. W. and Hurst, E. W. (1930): J. Path. and Bact., 33, p. 17.
Faulkner, J. M. and Taylor, F. H. L. (1937): Ann. int. Med., 10, p. 1867.
Giroud, P. (1936): Compt. Rend. Soc. Biol., Paris, 121, p 714.
Greenwald, C. K. and Harde, E. (1935): Proc. Soc. exp. Biol. Med., 32, p. 1157
Harde, E. and Benjamin, H. R. (1935): *Ibid.*, 32, p. 651.
Harde, E., Rothstein, I. A. and Ratish, H. D. (1935): *Ibid.*, 32, p. 1088.
Harris, L. J. and Ray, S. N. (1935): Lancet, 1, p. 71.
Harris, L. J., Abassy, M. A. and Yudkin, J. (1936): *Ibid.*, 1, p. 1488.
Harris, L. J., Passmore, R. and Pagel, W. (1937): *Ibid.*, 2, p. 183.
Henry, J. N. and Johnson, G. E. (1934): J. Amer. med. Ass., 103, p. 94
Hirszfeld, H., Hirszfeld, L. and Brokman, H. (1924): J. Immunol., 9, p. 571.
Holden, M. and Resnick, R. (1936): *Ibid.*, 31, p. 455.
Hurst, E. W. (1936): J. Path. and Bact., 42, p. 271.
Jungeblut, C. W. (1934): J. Immunol., 27, p. 17.
Jungeblut, C. W. (1935): J. exp. Med., 62, p. 517.
Jungeblut, C. W. (1936): Amer. J. med. Sci., 192, p. 661.
Jungeblut, C. W. (1937): J. exp. Med., 65, p. 127; and 66, p. 459.
Jungeblut, C. W. and Engle, E. T. (1932): J. Amer. med. Ass., 99, p. 2091.
Jungeblut, C. W. and Feiner, R. R. (1937): J. exp. Med., 66, p. 479.
Jungeblut, C. W., Meyer, K. and Engle, E. T. (1934): J. Immunol., 27, p. 43.
Jungeblut, C. W. and Zwemer, R. L. (1935): Proc. Soc. exp. Biol. Med., 32, p. 1229.
Lyman, C. M. and King, C. G. (1936): J. Pharm. exp. Ther., 56, p. 209.
Medical Research Council, Special Report Series, No. 167, 1932, pp. 28 and 193.
McCormick, W. J. (1938): Canad. med. Ass. J., 38, p. 260.
Ormerod, M. J. and Unkauf, B. M. (1937): *Ibid.*, 37, p. 134.
Ormerod, M. J., Unkauf, B. M. and White, F. D. (1937): *Ibid.*, 37, p. 268.
Pakter, J. and Schick, B. (1938): Amer. J. Dis. Children, 55, p 12

Poliomyelitis: The International Committee for the Study of Infantile Paralysis, Baltimore, 1932, pp. 316, 427, 448.
Sigal, A. and King, C. G. (1937): J. Pharm. exp. Ther., 59, p. 468.
Torrance, C. C. (1937): Proc. Soc. exp. Biol. Med., 35, p. 654.
Turner, A. J. (1938): Med. J. Austral., 1, p. 490.
Weisenburg, T. H. (1917): Med. Clin. N. Amer., 1, p. 393.
Wilkinson, J. F. and Ashford, C. A. (1936): Lancet, 2, p. 967.
Zingher, A. (1917): Amer. J. Dis. Children, 13, p. 247.

# THE EFFECT OF THE IONS OF COPPER, ZINC AND MERCURY ON THE BLOOD PIGMENT

by

WILLIAM ARTHUR RAWLINSON[1]

(From the Walter and Eliza Hall Institute, Melbourne).

*(Submitted for publication 27th July, 1938.)*

The fact that certain metallic ions interact with proteins often yielding precipitates has long been known. The phenomena were frequently included in the category of denaturation, and a summary of many of the earlier-known facts may be found in a text-book of biochemistry such as Brailsford Robertson's "The Physical Chemistry of Proteins" or among the monographs on proteins.

The conclusions of earlier workers were generally invalidated by the fact that the salts of many of the heavy metals are hydrolysed slightly by water yielding solutions with an acid reaction. The subject was also retarded by the application to proteins of the "electrical charge" outlook of colloid chemistry. While modern research on the questions of valency and chemical reaction may well lead back to an outlook with much in common with the earlier one there can be no gainsaying that the path to progress in protein chemistry was entered upon when the proteins were studied by the classical methods of organic and physical chemistry that had proved so powerful in dealing with simpler substances. Owing to limitations in methods of analysis very little could be ascertained beyond the bare facts that in approximately neutral solution the salts of many heavy metals such as silver, copper, lead, mercury or chromium reacted with proteins to form insoluble compounds which could be redissolved in acid or alkaline solutions. Perhaps no branch of biological chemistry has been more profoundly changed by the introduction of the means for the determination of the concentration of hydrogen ions than protein chemistry. Modern methods for the determination of small amounts of metals permit the subject to be further investigated, and the use of the glass-electrode places the all-important question of the hydrogen-ion concentration on a much surer basis. It is therefore somewhat surprising to find that of recent years there has been relatively little attention paid to the subject of the compounds of heavy metals with proteins.

The work described in this paper was undertaken in order to throw some light on the effect of the divalent ions of copper, zinc and mercury on the protein of the

[1] The work was carried out under a grant from the National Health and Medical Research Council.

blood pigment. It commenced with an examination of the precipitate of oxyhaemoglobin by small amounts of zinc salts, and was extended to include the salts of copper and mercury and on the protein side, globin, the native protein of the blood pigment.

## EXPERIMENTAL.

The first experiments consisted in determining the range of pH within which oxyhaemoglobin was precipitated by small amounts of zinc sulphate. The oxyhaemoglobin used was that of the rabbit.

*Preparation of Oxyhaemoglobin Solution.*

Fifteen c.c. of rabbit's blood were centrifuged and washed four times with 0·9 p.c. sodium chloride. Five c.c. of the erythrocytes obtained were then diluted to 30 c.c. with distilled water. One p.c. acetic acid was slowly added until the solution reached pH 5·7 (about 0·8 c.c. per 1 c.c. of cells). A glass-electrode was used to determine the pH. This adjustment to pH 5·7 had been found suitable for the purpose of separating the stroma from the oxyhaemoglobin. After cooling in the refrigerator the solution was centrifuged and the clear supernatant solution of oxyhaemoglobin pipetted off and diluted with water to yield a 2.5 p.c. solution. Protein concentration was accurately checked by coagulation and weighing. In a series of 20–30 small, clean test tubes of about 4 c.c. capacity were placed successively a measured volume of oxyhaemoglobin solution, varying amounts of 0·01 N sodium hydroxide, sufficient distilled water to make a total final volume of 3·0 c.c. and 1·0 c.c. of 0·0025 M zinc sulphate solution. The tubes were stoppered immediately and shaken gently to ensure complete mixture of the contents. The tubes were left to stand overnight at about 20°C. Next morning the tubes were centrifuged. A portion of the supernatant fluid was transferred directly into the cup of the glass-electrode outfit for the determination of pH, thus minimizing any risk of accidental change of pH of solutions so lightly buffered. The remainder of the supernatant fluids were transferred to another series of tubes for the determination of zinc concentration.

*Determination of Zinc.*

Dithizone (diphenylthiocarbazone) was found to be the most suitable reagent for the colorimetric determination of zinc ions. It dissolves in dilute sodium hydroxide solutions giving a deep orange colour. With zinc ions it gives a red colour or a cherry red precipitate at higher concentrations. The sample used by this author was impure and it was found necessary to purify it before quantitative results could be obtained. The original substance had a peculiar odour and when dissolved in dilute sodium hydroxide gave an opalescent solution the colour of which faded with great rapidity. The trouble was apparently due to oxidation products. Purification was carried out as follows:

The dithizone was dissolved in carbon tetrachloride (0·1 gm. per 50 c.c.). This solution was then extracted with 50 c.c. of 9 N ammonium hydroxide. The extraction was repeated twice. The dithizone was dissolved in the aqueous phase whilst the oxidation products remained in the carbon tetrachloride. The aqueous layers were combined and acidified with 5 N hydrochloric acid. The precipitate was filtered, washed well with water and dried *in vacuo* at room temperature. This purified reagent had no odour and gave a clear solution with sodium hydroxide. Much less of this reagent was necessary to give a suitable colour range with zinc ions.

For use 15 c.c. of 0·02 N sodium hydroxide was saturated with purified dithizone and filtered. This filtrate was diluted to about 130 c.c. The final concentration of dithizone was so chosen as to prevent the yellowish tinge of the reagent from interfering with the red colour due to its zinc compound, whilst at the same time an excess of dithizone was still present in the standard tube containing most zinc.

A series of tubes (§ in. diam.) was set up to serve as a set of standards containing 0·10; 0·20; 0·30; up to 0·80 c.c. of 0·00025 M zinc sulphate solution, 2·0 c.c. of the suitably diluted dithizone solution and distilled water to give a total volume of 4·0 c.c. By comparison with these standards concentrations of zinc from 0·00002 M upwards could be determined. A measured volume of the supernatant fluid of each tube was aded to 2·0 c.c. of the same dithizone solution and the volume made up to 4·0 c.c. with distilled water. The tube was then compared with the standard tubes until a match was obtained.

TABLE 1.

*Range of pH in which the Protein is Completely Precipitated.*

| Protein. | Metallic Ion. | Limits of pH. |
|---|---|---|
| Globin | Zinc | 7·5– 9·6 |
| Globin | Cupric | 7·0– 8·2 |
| Globin | Mercuric | 6·3– 9·0 |
| Oxyhaemoglobin | Zinc | 7·0– 8·1 |
| Oxyhaemoglobin | Cupric | 7·0– 8·3 |
| Oxyhaemoglobin | Mercuric | 7·0–10·0 |

*Mercury and Copper.*

The experiments on mercury and copper were carried out in an essentially similar manner using 0·5 c.c. of 0·01 M mercuric acetate and 0·5 c.c. of 0·01 M copper sulphate respectively. In each case the amount of salt used was the least that would readily cause complete precipitation of the protein at any pH used.

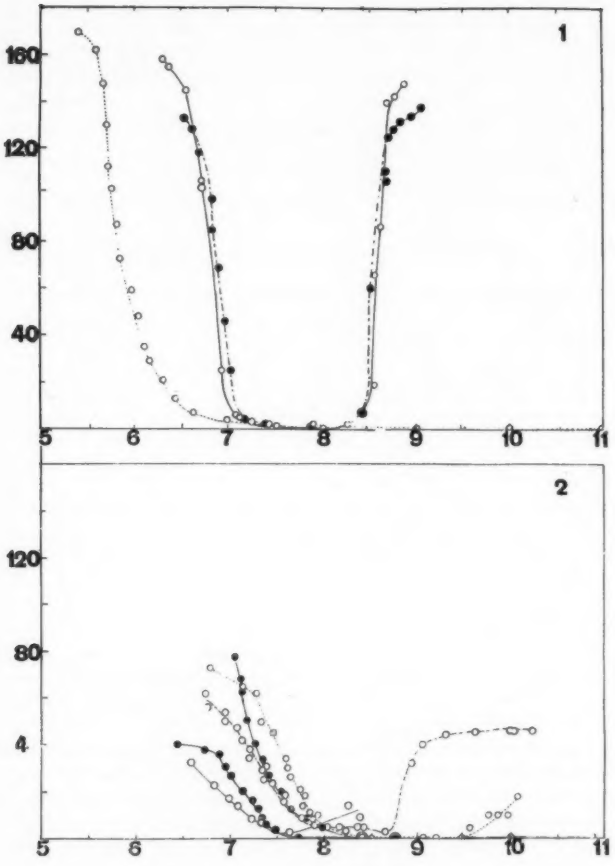
*Determination of Mercury.*

The reagent most recommended in the literature for colorimetric determination of mercuric ions was diphenylcarbazone. It formed a pale red solution in dilute sodium hydroxide and with mercuric ions gave a violet coloration at low concentrations. Although this reagent was extremely sensitive it was difficult to use for quantitative estimations primarily because of its rapid fading. Even after recrystallization from aqueous alcohol fading still occurred. Chloride ions also interfered with the intensity of its colour. These considerations suggested a return to dithizone which gave a similar colour with mercuric ions to that with zinc ions but with less intensity. By carefully standardizing the dilution of dithizone mercury could be determined with the same accuracy as zinc. The important point again was to dilute the dithizone sufficiently to reduce its yellow colour and also to give a suitable range of standard tubes, viz. 0·10; 0·20; 0·30 to 0·80 c.c. of 0·00025 M mercuric acetate solution. The acetate was used to avoid the interference of chloride ions and in all work with mercury the chloride concentration was kept to a minimum.

*Determination of Copper.*

The first reagent tried out for the colorimetric estimation of copper was dithio-oxamide (rubeanic acid). A solution was prepared by dissolving the reagent in a little alcohol and sodium hydroxide and diluting with water. The solution obtained was pale golden brown, and with copper ions it gave a green coloration. After a series of tests it was found that a standard copper sulphate solution of at least 0·0010 M was necessary to give a suitable range of colours.

Sodium diethyl dithiocarbamate was found to be more suitable. It formed a colourless solution in water and gave a yellow coloration with traces of copper ions. It was possible to make a suitable series of standard tubes as in the case of zinc, viz. 0·10; 0·20; 0·30 to 0·80 c.c. of 0·00025 M copper sulphate plus 1·0 c.c. of dilute reagent and finally diluted to 3·0 c.c. with distilled water.




**Figs. 1 and 2.**

Abscissae: pH.
Ordinates: M × $10^5$. M is Molarity of Supernatant solution.

**Fig. 1.**

- o - - - - o - - - - o - - Copper sulphate + water only.
- o ——— o ——— o —— Copper sulphate + oxyhaemoglobin.
- —●— - —●— - —●— - Copper sulphate + globin.

**Fig. 2.**

- o - - - - o - - - - o - - Zinc sulphate + globin.
- ●——— ●——— ●—— Zinc sulphate + water only.
- —o— - —o— - —o— - Zinc sulphate + oxyhaemoglobin.
- o ——— o ——— o —— Zinc sulphate + oxyhaemoglobin.

Similar experiments were performed on the effect of these three heavy metal ions on globin, the native protein of the blood pigment. The globin was prepared from ox corpuscles by the acid-acetone method of Anson and Mirsky (1929). In this case however the stroma was not removed before the removal of the haematin, but was precipitated during the removal of the denatured protein. After removal of the denatured protein the globin solution was adjusted to pH 6·0. The concentration of the protein was determined by coagulation in the presence of formalin (Bick, 1936). The solution used in experiments was 1·55 p.c.

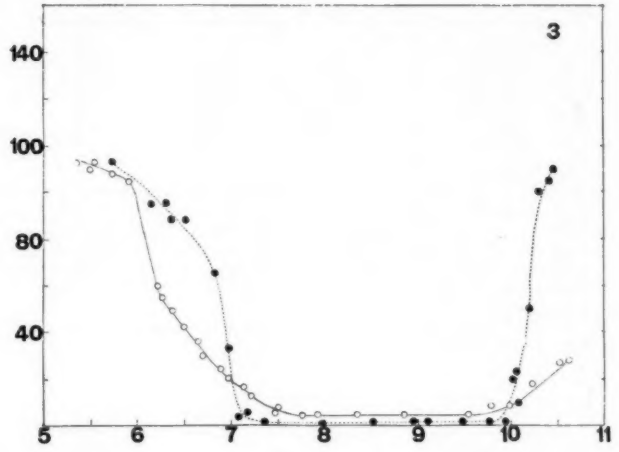



Fig. 3.

– – ● – – – ● – – – ● – – Mercuric acetate + oxyhaemoglobin.
——o——o——o—— Mercuric acetate + globin.

*The Nature of Precipitates Obtained.*

The precipitate of the zinc-oxyhaemoglobin compound was of a reddish colour, readily soluble in either dilute acetic acid or dilute ammonia. In either case the protein appeared to have suffered no change as a result of precipitation. It showed the normal spectrum of oxyhaemoglobin as judged by the Hartridge reversion spectrometer and the oxygen could be removed by repeated equilibration with pure hydrogen. In addition the oxygen could be washed away from the precipitate by means of pure hydrogen without altering the solubility of the protein zinc compound. In fact this compound conforms to the definition of the native blood pigment as judged by its reversible combination with oxygen and to the definition of an irrenaturable meta-protein as judged by its solubility relationships. The zinc compound could be kept at room temperature for several hours and at 0° C. for months without much of it losing the specific property of reversibly combining with oxygen.

The mercuric compound, if precipitated with the minimal amount of mercuri acetate, remains apparently unchanged for some time. If, however, a large exces of salt be present the pigment undergoes some apparent change analogous to th formation of parahaematin.

TABLE 2.

| Graph No. | Oxyhaemoglobin. Mg. per c.c. | Metallic Ion. | Mg. per c.c. | Time of standing before dilution (mins.). | pH. |
|---|---|---|---|---|---|
| 4 | 0·111 | — | — | 0 | 4·5 |
| 5 | 0·111 | Zinc | 0·013 | 95 | 4·9 |
| 6 | 0·111 | Mercuric | 0·040 | 60 | 3·9 |
| 7 | 0·111 | Mercuric | 0·040 | 60 | 4·3 |
| 8 (a) | 0·111 | Cupric | 0·010 | 20 | 4·7 |
| 8 (b) | 0·111 | Cupric | 0·016 | 0 | 5·4 |

As an example No. 5 was carried out as follows:

0·15 c.c. of 0·74 p.c. oxyhaemoglobin solution was pipetted into a standard flask followed by 0·10 c.c. of 0·05 N ammonium hydroxide and washed in with 1 c.c. of distilled water. Then 0·20 c.c. of 0·01 M zinc sulphate solution was added, a further 1 c.c. of water and the contents mixed. The protein was precipitated and allowed to remain thus for 95 minutes. Then 0·05 c.c. of 1 p.c. acetic acid was added to redissolve the precipitate and the contents diluted to 10 c.c. with water. This solution was photographed in a 1 cm. tube. The control tube contained all the reagents except the pigment.

In No. 8 (b) however the oxyhaemoglobin was allowed to come in contact with the copper ions before the addition of water.

TABLE 3.

| Graph No. | Globin. Mg. per c.c. | Haemin. Mg. per c.c. | Metallic Ion. | Mg. per c.c. | Time of standing before dilution (mins.). | pH. |
|---|---|---|---|---|---|---|
| 9 | 0·44 | 0·0036 | — | — | 0 | |
| 10 | 0·44 | 0·0036 | Zinc | 0·013 | 0 | 5·4 |
| 11 | 0·44 | 0·0036 | Mercuric | 0·020 | 0 | 4·8 |
| 12 | 0·44 | 0·0036 | Cupric | 0·011 | 0 | 4·9 |
| 13 | 0·44 | 0·0036 | Cupric | 0·011 | 30 | 4·9 |

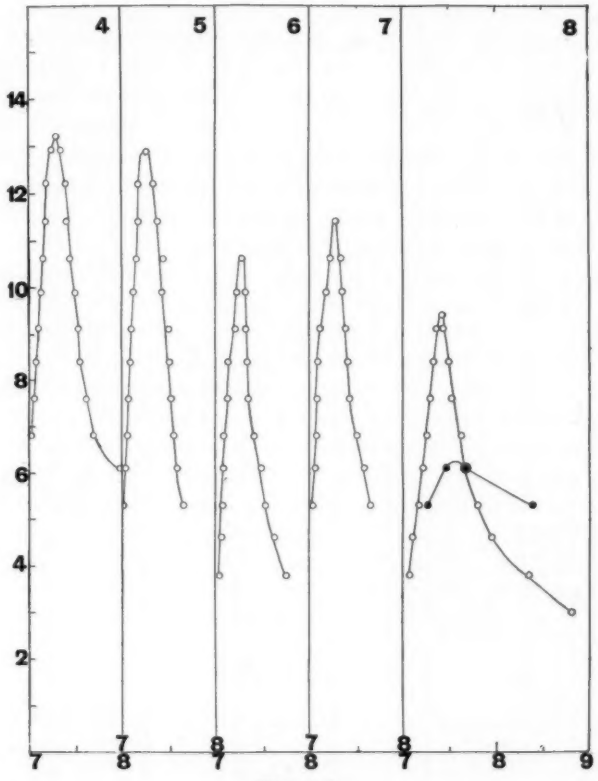
As an example No. 13 was carried out as follows: 0·80 c.c. of 0·55 p.c. globin solution was pipetted into a standard flask followed by 0·15 c.c. of 0·6 N ammonium hydroxide and 0·80 c.c. of 0·0025 M copper sulphate solution. The protein was precipitated and allowed to remain thus for 30 minutes. Then 1·10 c.c. of 1 p.c. acetic acid was added to redissolve the precipitate, 0·50 c.c. of haemin solution and the content diluted to 10 c.c. with water. The control tube contained all the reagents except the haemin.

The copper-oxyhaemoglobin compound is almost immediately changed even when precipitated in solutions containing as little copper salt as possible and a brownish substance is formed. This on solution in alkali has a spectrum somewhat like that of alkaline haematin, and on reduction with sodium hydrosulphite the spectrum is that of haemochromagen.

A larger specimen of the zinc-oxyhaemoglobin compound was prepared as follows:

In a litre flask were placed 40 c.c. of 2·5 p.c. oxyhaemyglobin solution and 80 c.c. of 0·01 N sodium hydroxide diluted to 280 c.c. with distilled water. After mixing 160 c.c. of 0·0025 M zinc

sulphate solution was added drop by drop with continuous stirring. The protein slowly precipitated and the mixture was allowed to stand overnight at 20°C. The pH was 7·5. Next day the precipitate was separated by centrifuging, well washed with water, suspended in water and left in the refrigerator. This washing was repeated each day for a week. Between washings the precipitate was left in water in the refrigerator. At the end of a week no zinc could be detected in the supernatant fluid. It was then left undisturbed for a week, during which period a small trace of




**Figs. 4–8.**

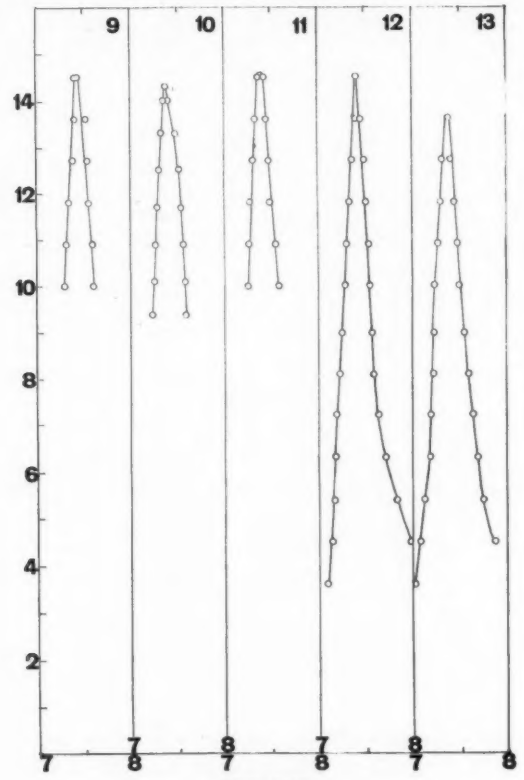
**(See Table 2.)**

Abscissae: $\nu 10^{-14}$ $\nu$ is frequency per second.
Ordinates: $\epsilon 10^{-4}$

Where $\epsilon = \frac{M}{cd} \log \frac{I_0}{I}$

C = No. of grams of substance in one litre of solution.
d = Depth of cell in cm.
$I_0$ = Intensity of incident beam.
I = Intensity of emergent beam.
M = Equivalent weight. For haemin, M = 652.
For oxyhaemoglobin, M = 16,700.

oxyhaemoglobin redissolved in the fluid. The liquid slowly became frozen and the bulky precipitate started to decrease in volume. After another week a compact mass settled out on thawing. It had a vitreous appearance and a very dark colour. On standing in distilled water dissociation occurred very slowly and traces of both oxyhaemoglobin and zinc could be detected in the supernatant fluid. The compacted precipitate on drying *in vacuo* became very dark, almost black and shining. The undried dark red substance, though insoluble in water readily dissolved in dilute ammonia to a clear solution whose spectrum was that of oxyhaemoglobin.




**Figs. 9–13.**
**(See Table 3.)**

This procedure was repeated in the case of native globin and zinc ions. The well washed precipitate was allowed to stand in the refrigerator and the liquid became frozen whilst the precipitate slowly contracted until after ten days a compact, clear and vitreous solid settled out on thawing.

Although these "compacted" precipitates have a vitreous lustre no definite crystal shape can be detected under the microscope and the change is most probably

due to the removal of water from the protein-metal precipitate on freezing. An X-ray diagram should give further information on this point.

The ultra-violet spectro-photometer was used to confirm the observations with the visible spectroscope concerning the condition of oxyhaemoglobin and of globin after treatment with the salts of the above heavy metals. For this purpose specimens of oxyhaemoglobin were precipitated with zinc sulphate, mercuric acetate and copper sulphate, and the precipitates after standing a short time were re-dissolved in dilute acetic acid and diluted to a convenient concentration for photography. The spectra obtained showed that in the case of zinc no measurable amount of change had occurred, while with mercury some, and with copper almost all of the blood pigment was changed.

With globin, the precipitate was dissolved in dilute acetic acid, a solution of haemin dissolved in dilute ammonia added and the reaction of the solution adjusted to pH 4·8 with acetic acid before photographing. This reaction had been found previously (Holden, 1936) to be suitable for the photography of methaemoglobin.

The oxyhaemoglobin-zinc precipitate as dissolved for photography had a pH 4·9. A specimen of the mercuric compound dissolved at pH 3·9 showed less unchanged oxyhaemoglobin than that at pH 4·35, doubtless because of the increased acidity. At reactions less acid the mercuric compound dissolved with difficulty. It is thus probable that some at least of the relatively small amount of change observed in the case of mercury may be due to the method necessary for solution of the oxyhaemoglobin compound rather than to any direct action of the mercuric salt. Both the visual and the ultra-violet observations indicated that whereas zinc and mercury salts precipitate both globin and oxyhaemoglobin with little irreversible change, copper salts precipitate globin only in this manner while they alter oxyhaemoglobin to a compound resembling parahaematin. By precipitating oxyhaemoglobin in low concentration with the minimum amount of copper sulphate and re-dissolving rapidly some methaemoglobin can be recovered.

## DISCUSSION.

The chief facts observed in the course of this work are as follows:

1. A definite zone of hydrogen-ion concentration exists for the precipitation of the protein by the metallic ion, which depends on the nature of both. The amounts of the different ions necessary for complete precipitation appear to be about the same in the case of copper and mercury, i.e. about 10–12 gram atoms per gram equivalent of oxyhaemoglobin, and in the case of zinc about one-quarter of this amount suffices. Indirect calculations of the precise amount of metallic ion combined with the protein are unprofitable owing to some uncertainty with regard to the status of the salt not so combined. This is demonstrated on studying the pH-solubility curves for zinc sulphate in water only. Had there been a simple solubility relationship the curve for the lower initial zinc concentration should

have been the same as for the higher concentration solution except for that portion above the initial concentration of the dilute solution.

2. No clear relation exists between the solubility of a metallic salt in the presence of the protein tested and its solubility in water at the same hydrogen-ion concentration. Thus in the case of mercury no precipitation occurs in water between the limits of pH tested. For zinc sulphate the solubility curve in water was higher on the acid side than in the presence of the protein and approaching the latter at the pH range of precipitation of the zinc-oxyhaemoglobin compound. In the case of copper the solubility on both sides of the precipitation range was less in the absence of protein than in its presence. The zinc-oxyhaemoglobin compound re-dissolves on the alkaline side of neutrality and above pH 8·5 about half the zinc remains in solution leaving the other half precipitated.

3. It is interesting to note that copper alone has the same range of precipitation for globin as for oxyhaemoglobin which it alters. Since the visual spectra of the altered substance and that formed from it by the action of an alkaline-reducing agent correspond to the presence of denatured globin-parahaematin and of denatured globin-haemochromagen respectively, and since globin and denatured globin have the same iso-electric point, it is tempting to suggest that the copper ions are attached to corresponding points on the molecules. The fact that copper has an immediate irreversible effect on the blood pigment while it has no such effect on globin, does not seem at present open to any explanation. It has been shown (Holden, 1936, 1937) that denaturation of oxyhaemoglobin by acid, alcohol or sodium salicylate is essentially an oxidation reaction, coupled to the oxidation of the prosthetic group. Since the cupric ion does not appear to oxidize globin it would seem probable that it must oxidize the ferrous atom in the reduced haematin in oxyhaemoglobin and that in some way the oxidative denaturation of the globin is coupled to the oxidation of the iron. The denaturation by cupric ions is effected on both sides of the precipitation range, e.g. pH 5–6 and 8–9, which is an indication that with this reagent the reactions of denaturation and precipitation are not necessarily closely linked together.

## SUMMARY.

Oxyhaemoglobin can be precipitated by zinc sulphate without interfering with its power to combine reversibly with oxygen. With mercury a little of the oxyhaemoglobin is altered and with copper it is all changed to a parahaematin type of compound. Globin can be precipitated with zinc, mercuric and copper salts without undergoing any change that affects its ability to form methaemoglobin. The methaemoglobin from zinc-globin or mercuric-globin can be converted to oxyhaemoglobin. The physical properties of the metal-globins and metal-oxyhaemoglobins described would cause them to be included in the category of meta-proteins.

## ACKNOWLEDGMENT.

This work, as part of a thesis for a senior degree, was carried out in Mr. H. F. Holden's laboratory under the general direction of Professor W. J. Young, of the University of Melbourne. The author wishes to thank Professor Young and Mr. Holden for their interest and encouragement.

## REFERENCES.

Anson, M. L. and Mirsky, A. E. (1929): J. Gen. Physiol., 13, p. 469.
Bick, M. (1936): Austral. J. exp. Biol., 14, p. 305.
Galeotti, G (1904): Zeit. Physiol. Chem., 40, p. 492.
Harnack, E. (1881): *Ibid.*, 5, p. 198.
Holden, H. F. (1936): Austral. J. exp. Biol., 14, p. 291.
Holden, H. F. (1937): *Ibid.*, 15, p. 43.
Pauli, W. (1905): Beit. Chem. Physiol. Path., 6, p. 233.
Robertson, T. B.: The Physical Chemistry of the Proteins.

# VARIATIONS IN THE BEHAVIOUR OF PARETIC CEREBROSPINAL FLUIDS WITH DIFFERENT TYPES OF GOLD SOLS

by

S. W. PENNYCUICK, C. E. WOOLCOCK AND R. J. COWAN

(From the Johnson Chemical Laboratories, University of Adelaide, and the Institute of Medical and Veterinary Science, Adelaide).

*(Submitted for publication 15th September, 1938.)*

The routine preparation of gold sols has become of special importance since Lange (1912) showed the value of these sols in the diagnosis of certain pathological conditions. The cerebrospinal fluid of healthy persons does not affect stable gold sols, whereas pathological fluids produce a graded series of coagulative effects. This behaviour, in conjunction with other tests, has proved so valuable a diagnostic aid, that it is now used as a standard test in most pathological laboratories.

As a result, the demand for stable gold sols has assumed large proportions. Nevertheless, the sol is usually prepared on a relatively small scale in each laboratory, and hence a routine preparation is desired which is both simple and precise. Experience has shown, however, that the actual preparation of the sol is by no means the sole consideration; it is necessary also to ensure that it will be of the desired sensitivity, so that it will respond at all times to the rather delicate requirements of the Lange test.

In a former publication (Pennycuick, Woolcock and Cowan, 1938) the authors recommended the use of a formaldehyde-reduced sol, prepared by seeding the solution with the requisite number of gold nuclei, and having the sensitivity adjusted by varying the pH.

The work has now been advanced by an investigation into the preparation and suitability of other gold sols, namely the oxalate-reduced sol, the phosphorus-reduced sol and the Bredig gold sol.

Furthermore, following the results of Pennycuick and Woolcock (1938), work has also been carried out on the corrective influence exerted by small amounts of stannic chloride on the water used in the preparation of gold sols. Stannic chloride has the effect of checking the harmful action of the inhibitory material in unsuitable water, and thereby facilitating the production of the sol.

*Oxalate-reduced Gold Sols.*

Potassium oxalate or oxalic acid has sometimes been recommended as a reducing agent to replace formaldehyde in the preparation of gold sols suitable for the Lange test [Patterson (1931), Williams (1935) and Sutherland (1937)].

Variations in the method of preparation have been introduced from time to time, but the method adopted in this work has been the normal one, namely the addition of 1 c.c. of 1 p.c. auric chloride, drop by drop, with constant stirring, to a boiling solution of 0·01 p.c. of neutral potassium oxalate. The gold chloride used was the acid salt, and accordingly the preparation was carried out in slightly acid solution.

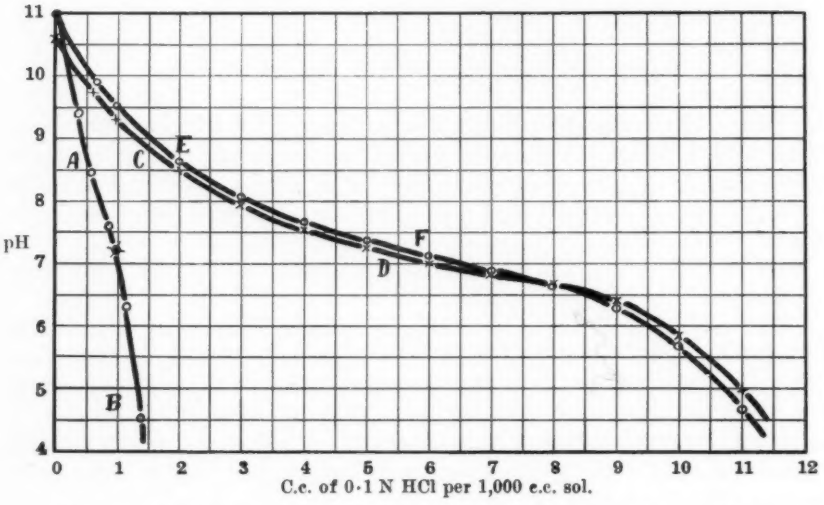



**Figure 1.** *Titration curves of oxalate-reduced and formaldehyde-reduced gold sols.*
A.B. Oxalate-reduced sol (unbuffered).
C.D. Oxalate-reduced sol (buffered).
E.F. Formaldehyde-reduced sol.

This method gives a clear fine-grained sol in water which normally is unsuitable for the production of formaldehyde-reduced sols. In fact, it is one of the easiest of all gold sols to prepare, provided that the solution is kept on the acid side of neutral. If the pH is raised above 7, the technique of preparation must be varied (see later).

Our investigations show that the chief weakness in the oxalate-reduced sol lies in its unbuffered state. This is revealed by the course of the titration curve, represented by AB in Fig. 1, where the upper part of the curve is obtained by titrating the sol with sodium carbonate, and the lower part with hydrochloric acid.

The titration figures are set out in Table 1, the pH readings being taken with the glass electrode. The slope of the curve shows that the sol is unbuffered.

Now, the pH values of the freshly-prepared sols are low, lying between 5·3 and 6·5; and, as is the case with all gold sols of low pH, these sols are relatively unstable, and their reactions towards paretic cerebrospinal fluids are too sensitive. For example, paretic fluids which should give the normal figures 5555321000, show readings of 5555555432 with freshly prepared oxalate-reduced sols.

In order to obtain the correct response, it is necessary to reduce the sensitivity of the sol by increasing the pH, say by the addition of a little potassium hydroxide; but this cannot be done with any precision because of the unbuffered state of the sol.

The difficulty can be overcome by the direct addition of a buffering agent, particularly one which raises the pH and thereby increases the stability; but care is necessary in order to avoid the introduction of excess electrolyte which may alter the conditions of the test.

TABLE 1.

*Titration of Gold Sols with HCl.*

| C.c. of 0·1N HCl per 1,000 c.c. sol. | Oxalate-reduced (unbuffered). pH | Oxalate-reduced (buffered). pH | Formaldehyde-reduced. pH |
|---|---|---|---|
| 0 | 9·0 | 8·71 | 9·00 |
| ·2 | 8·68 | — | — |
| ·4 | 7·88 | — | — |
| ·6 | 7·31 | 8·15 | 8·29 |
| ·8 | 6·69 | — | — |
| 1·0 | 6·47 | 7·84 | 8·03 |
| 1·2 | 5·78 | — | — |
| 2·0 | 3·81 | 7·34 | 7·33 |
| 3·0 | 3·55 | 6·96 | 7·01 |
| 4·0 | — | 6·67 | 6·79 |
| 5·0 | — | 6·50 | 6·58 |
| 6·0 | — | 6·36 | 6·40 |
| 7·0 | — | 6·25 | 6·26 |
| 8·0 | — | 6·11 | 6·09 |
| 9·0 | — | 5·88 | 5·82 |
| 10·0 | — | 5·52 | 5·46 |
| 11·0 | — | 4·97 | 4·74 |

The buffering agent can be added either before or after the preparation of the sol. In the former case, a small amount of potassium carbonate, sufficient to bring the initial pH of the oxalate solution, before reduction, to about 10, answers the purpose; but as the pH is now high, the normal method of preparation does not always yield satisfactory sols, and some variation is desirable, such as the addition of auric chloride in the cold and the subsequent raising of the temperature to the boiling point (Patterson, 1931). However, once obtained, these sols are found to be well buffered and quite stable. Indeed, they are generally so stable that they fail to give the necessary precipitations with paretic cerebrospinal fluids; but their sensitivity can now be adjusted with some precision by the addition of

the requisite amount of hydrochloric acid as recommended by Pennycuick, Woolcock and Cowan (1938) for formaldehyde-reduced sols. The adjustment can be carried out until the sol is brought within the sensitive range, or better, until a sample of the sol gives the correct response with a known paretic fluid.

Although this treatment is fairly satisfactory, the authors have found that the better method is to add the buffering agent after the sol has been prepared. This method is more convenient and more reliable. As a buffering agent, 1 c.c. of 0·25 N $KHCO_3$ per 100 c.c. of sol, will produce the necessary effect, at the same time raising the pH and increasing the stability. The highly buffered state of such a sol is shown by the course of the titration curve, represented by CD in Fig. 1, where hydrochloric acid is used as the titrating agent. The corresponding figures are given in Table 1.

The pH of the sol is now in the neighbourhood of 8·5, which is too high for the Lange test, but it can be adjusted and the sensitivity increased by the addition of hydrochloric acid. Furthermore, the titration curve (CD) corresponds so closely with the curve for the formaldehyde-reduced sol (shown by EF in Fig. 1), that the amounts of hydrochloric acid necessary to sensitize the sols are the same in each case. Accordingly, the figures given by Pennycuick, Woolcock and Cowan (1938) for sensitizing the formaldehyde-reduced sol, may be applied to the oxalate-reduced sol without alteration.

There is, however, one difference in reaction between oxalate- and formaldehyde-reduced sols which is always evident, despite the similarity in the titration curves. The oxalate-reduced sols do not show the same sensitive pH range with paretic cerebrospinal fluids as is shown by the formaldehyde sols. Whereas the latter range is well defined, lying between pH 6·5 and 7·3, the oxalate-reduced sols show a much narrower range, and one moreover which varies from preparation to preparation. For example, some preparations show a sensitive range between 6·3 and 6·8, whilst others show a range as high as 7·5 to 7·9. Accordingly, the only satisfactory method of sensitization is to add hydrochloric acid until a sample of the sol gives the correct response with a known paretic fluid.

Formaldehyde-reduced sols also show characteristic gradations in the Lange response with varying pH (Pennycuick, Woolcock and Cowan, 1938) which gives them special precision; the fact that oxalate-reduced sols do not give the same regular response serves to emphasize the difference in surface structure of the gold particles in the two preparations.

*Phosphorus-reduced Gold Sols.*

The phosphorus-reduced sols were prepared by reducing an alkaline solution of auric chloride with phosphorus dissolved in ether. Details of the preparation are given by Pennycuick, Woolcock and Cowan (1938). Before use they were diluted with an equal volume of water in order to make the gold content comparable with that of the other sols used in this work.

This sol forms quite readily in once-distilled water, and no particular precautions are necessary. The pH of the product is found to lie between 7·3 and 8·3, and titration with hydrochloric acid reveals that the sols are moderately well buffered.

These sols are usually fine-grained, homo-dispersed and very stable. They have occasionally been recommended for use in the Lange test, for example by Ettisch and Einstein (1931). Our results, however, whilst confirming their remarkable stability, show that in general they are not suitable for use with paretic fluids.

TABLE 2.

*Variations in Lange Response Shown by Formaldehyde-reduced Sols and Phosphorus-reduced Sols.*

| pH of Sol. | Formaldehyde-reduced. | Phosphorus-reduced. |
|---|---|---|
| 7·5 | 5543320000 | 1110000000 |
| 6·8 | 5554321000 | 1111000000 |
| 6·3 | 5555321000 | 1111100000 |
| 5·8 | 5555532100 | 1111000000 |
| 5·4 | 5555543210 | 1122000000 |

Although the pH of the freshly-prepared sol is not unduly high (7·3 to 8·3), it exhibits an abnormal stability in the presence of paretic fluids, a stability which would only be associated with an abnormally high pH. This behaviour is shown by the results in Table 2, where the reactions at varying pH are set out. For comparison the corresponding results of a formaldehyde-reduced sol are also given.

From the table it is seen that at pH 7·5 neither sol is sufficiently sensitive, but whereas the formaldehyde-reduced sol shows a reaction slightly below normal, the phosphorus-reduced sol shows scarcely any response at all. The difference is accentuated as the pH is decreased. The formaldehyde-reduced sol shows a graded sensitivity, passing through the useful range between 6·3 and 6·8, but the phosphorus-reduced sol shows a bare minimum of response, even when the pH is reduced as low as 5·4.

If the pH is lowered still further, the phosphorus-reduced sol shows a sudden increase in sensitivity and ultimately gives an approximation to the Lange figures, but as it has now passed out of the buffered range, the figures are unreliable and difficult to reproduce.

One of the outstanding characteristics of this particular sol is the extremely small size of the particles. The exact dimensions cannot be determined, since the particles are amicrons and appear in the ultramicroscope as a diffuse cloud. A rough estimation showed that the diameters of the particles in many of our sols were of the order 3 to 4 mμ. The fact that these particles maintain their small dimensions during the reduction process indicates that there is an abnormally active stabilizing agent present; it apparently continues to exercise its strong protective action when the sol is exposed to the precipitating effect of paretic fluids.

*Bredig Gold Sols.*

Bredig gold sols are not usually regarded as being suitable for the Lange test although their use has been suggested by Manheims and Bernhard (1925). Our work shows that although these sols can be adapted for use they have no special features to recommend them; accordingly a brief summary only of our results will be sufficient.

The sols were prepared in 0·0001 N. solutions of caustic soda, potassium chloride or hydrochloric acid. As is indicated by the method of preparation, they were all found to be unbuffered, and they gave a variety of reactions which varied with the pH of the sol. On addition of a suitable buffering agent, each sol could be sensitized by adjusting the pH until a fairly satisfactory response was obtained against paretic fluids, but the sensitive range varied so much with the different preparations that difficulty was experienced in producing the same conditions. Owing to this weakness, together with the inconvenient method of preparation, the Bredig sol cannot compare with the formaldehyde- or the oxalate-reduced sols.

*The Effect of Seeding the Formaldehyde-reduced Sol with Tannic Acid.*

A small amount of phosphorus-reduced sol has been recommended by Pennycuick, Woolcock and Cowan (1938) as a seeding agent in the routine preparation of formaldehyde-reduced sols for use in the Lange test. From the fact that tannic acid itself rapidly forms fine-grained sols whose particles approximate in size to those of the phosphorus-reduced sol, it appeared to the authors that a small amount of tannic acid might serve the same purpose, and provide nuclei upon which the formaldehyde-reduced gold could build. The success of the action depends upon the well-known fact that tannic acid reduces gold chloride much more readily than does formaldehyde.

Accordingly, tests were carried out using distilled water which failed to give a suitable red sol with formaldehyde alone. The conditions were exactly the same as those described by Pennycuick, Woolcock and Cowan (1938) when seeding with a little phosphorus-reduced sol, the latter, of course, now being replaced by a small amount of tannic acid. In general the results were very satisfactory. Unsuitable water, which initially was quite useless for the preparation of a formaldehyde-reduced sol, now gave clear useful sols, which showed the characteristics of formaldehyde-reduced sols when prepared in highly purified water; they gave the same titration curves, and satisfactory results with paretic cerebrospinal fluids.

One rather remarkable feature was the extremely small amount of tannic acid necessary to bring about the corrective effect, an amount which varied with the condition of the water, but which, in general, lay between one part in 4,000,000 of water and one part in 40,000,000.

With the same quality water, different amounts of tannic acid frequently produced sols which were not strictly comparable, some being more sensitive than others. It was considered that this was due to the fact that different amounts of tannic acid formed different numbers of nuclei, which in turn caused a difference in the number and size of the particles in the finished sol. This, however, was not borne out by ultramicroscopic measurements, since the diameters of the particles did not show any regular sequence with increasing amounts of tannic acid.

Tests with paretic fluids showed that the best amount of tannic acid to use was the minimum necessary to correct the particular sample of water in hand. Such preparations, whilst fairly satisfactory, were not as consistently reliable as those prepared with the phosphorus-reduced sol as seeding agent. In the latter preparation, a definite number of nuclei can be introduced at any time, and this number can be varied as desired by varying the amount of seeding material; but with the tannic acid there is no indication of the number of nuclei formed, nor is there any evidence that by increasing the amount of tannic acid the number of nuclei will be increased in the same proportion.

*The Corrective Influence of Small Amounts of Stannic Chloride.*

It has been shown by Pennycuick and Woolcock (1938) that the inhibitory material in once-distilled water which prevents the formation of fine-grained red sols consists of positively charged hydrophobic colloids which are carried over in small amounts during the distillation. The harmful matter can be removed by adjusting the pH to 10, and adding a very small amount of stannic chloride (one part in 10,000,000). Apparently the colloidal stannic hydroxide formed by hydrolysis is peptized by the free base to form negatively charged particles, which react with the positively charged inhibitory material and so precipitate it. Water which ordinarily is unsuitable for the production of stable sols can be corrected by this treatment.

The method is applicable to the preparation of all gold sols, but is particularly useful in the formation of formaldehyde-reduced sols, these being the most susceptible to traces of harmful material. A technique was accordingly developed by which the stannic chloride could be added to the water either during the distillation or when the distillation was complete.

At first it appeared that the use of stannic chloride offered a valuable safeguard in the preparation of gold sols; for not only were these sols easily and regularly formed in water which normally would be considered unsuitable, but they gave excellent results with paretic cerebrospinal fluids. But in the final tests, when their behaviour with normal fluids was being checked, they showed a peculiar irregularity. Instead of the zero figures, 0000000000, they gave readings which were difficult to interpret, but which approximated to 0010111000. This variation, though small, was quite definite, and as it only appeared in water which had been

treated with stannic chloride, it was probably due to residual traces of colloidal stannic hydroxide. By using less stannic chloride, the effect sometimes disappeared, but as it could not be removed with any certainty, the method could not be adopted for regular use.

## SUMMARY.

The oxalate-reduced gold sol can be used in the Lange test if a buffering agent is added to the solution either before or after the reduction of the gold salt. In general, however, it is not as reliable as the formaldehyde-reduced sol.

The phosphorus-reduced gold sol cannot be used with any degree of precision.

The Bredig gold sol can be adapted for use, but its reactions are variable and its preparation is inconvenient.

A trace of stannic chloride when added to distilled water facilitates the formation of gold sols, but such sols, whilst suitable for paretic cerebrospinal fluids, show slight irregularities with normal fluids.

## ACKNOWLEDGMENT.

The authors are indebted to the Trustees of the Endowment Fund of the Council for Scientific and Industrial Research of the Commonwealth of Australia for a grant for the purchase of the gold used in this work.

## REFERENCES.

Ettisch, Von G. and Einstein, O. (1931): Naturwissenschaften, 19, p. 506.
Lange, Carl (1912): Berliner Klinische Wochenschrift, 49, p. 897.
Lange, Carl (1912): Z. Chemotherapie, 1, p. 44.
Manheims, P. J. and Bernhard, A. (1925): J. Lab. Clin. Med., 11, p. 235.
Patterson, J. (1931): Brit. J. exp. path., 12, p. 143.
Pennycuick, S. W. and Woolcock, C. E. (1937): Austral. J. exp. Biol., 15, p. 423.
Pennycuick, S. W. and Woolcock, C. E. (1938): In course of publication.
Pennycuick, S. W., Woolcock, C. E. and Cowan, R. J. (1938): J. Path. and Bact., 46, 3, p. 549.
Sutherland, J. (1937): Med. J. Austral., 11, p. 172.
Williams, W. C. (1935): J. Lab. Clin. Med., 20, p. 545.

# STUDIES IN THE PHYSIOLOGY OF PLANT PATHOGENIC BACTERIA

## THE FOOD REQUIREMENTS OF A XYLEM INVADER, *BACTERIUM SOLANACEARUM* E.F.S., AND OF A PHLOEM INVADER, *APLANOBACTER MICHIGANENSE* E.F.S.

by

ROSE MUSHIN

(From the School of Botany, University of Melbourne).

*(Submitted for publication 21st June, 1938.)*

## INTRODUCTION.

In the course of a series of studies on stimulation effects in plants and on the physiology of selected host plants in relation to parasitic infection by *Bacterium solanacearum* E.F.S. and *Aplanobacter michiganense* E.F.S. (Grieve, 1938), it became evident that an investigation of the food requirements of these organisms might prove of value. The former invades the xylem vessels in host plants such as tomato *(Lycopersicum esculentum)* and potato *(Solanum tuberosum)*, while the latter invades the phloem in tomato. This statement applies to the earlier stages of disease, since both organisms may break down other tissues as the disease progresses in each case.

It appeared that a study and comparison of the food requirements of these two vascular invaders might allow of an insight into their modes of parasitism and possibly throw some light on the question of solute migration in the xylem and phloem.

## HISTORICAL SURVEY.

The Brown Rot of Solanaceae caused by *Bacterium solanacearum* E.F.S. was described by Erwin F. Smith (1896). Various contributions bearing on its pathogenicity, host range, and cultural and physiological characters have appeared since, but only Honing (1912) has concerned himself specifically with the carbon and nitrogen requirements of *B. solanacearum*.

In Australia the disease was reported by Tryon (1894), McAlpine (1911) and Darnell-Smith (1918), but the causal organism was definitely identified and described by Grieve (1935). The organism he worked with in Victoria was regarded as a local strain.

The Grand Rapids disease caused by *Aplanobacter michiganense* E.F.S. was first described in America by Smith (1910). In Australia, Samuel (1926) was the first one to report a similar disease, but a full account of it was given by Magee (1935) during an outbreak of this disease in New South Wales and by Fish and Pugsley (1936) in Victoria. So far as the author is aware no full account of the food requirements of the organism has been published, although Stapp (1930) supplied some data on the subject.

## METHODS AND TECHNIQUE.

### *Source of Cultures.*

*B. solanacearum* was isolated from diseased tomato and potato plants in Victoria while *A. michiganense* was obtained from a stock culture also isolated in Victoria.

### *Media.*

Synthetic media were prepared containing different sources of carbon and nitrogen. Preference was given to compounds which are likely to be found in the xylem and phloem of host plants. The method of making up media and the technique of experiments were based on the work of Lewis (1930).

Each medium contained a standard mineral solution, which consisted of:

| | |
|---|---|
| Dibasic potassium phosphate | 0·31 per cent. |
| Monobasic potassium phosphate | 0·08 „ „ |
| Potassium chloride | 0·02 „ „ |
| Magnesium sulphate | 0·02 „ „ |

Whenever ammonia was the only source of nitrogen 0·25 p.c. of sodium ammonium phosphate replaced the potassium phosphates.

The other compounds were used in following quantities:

| | |
|---|---|
| Carbohydrates and alcohols | 0·5 per cent. |
| Asparagine, peptone, glutamic acid | 0·3 „ „ |
| Sodium salts of organic acids | 0·2 „ „ |
| Potassium nitrate and nitrite | 0·2 „ „ |
| Tyrosine | 0·1 „ „ |

All media were adjusted to pH 6·8 and distributed into test tubes in 6 ml. quantities. Sugar media were steamed on three successive days while other media were autoclaved.

All media were liquid and each contained the standard mineral solution with addition of one compound containing both carbon and nitrogen or two compounds, one to serve as a source of carbon and the other as a source of nitrogen.

### *Technique.*

*B. solanacearum* and *A. michiganense* were grown on agar slopes for 48 hours, then the cultures were emulsified with about 3 ml. of sterile distilled water and one loopful of the emulsion transferred to each medium.

In order to minimize the possibility of contamination only five transfers to synthetic culture media were made from one tube of bacterial emulsion, which was then discarded.

Growth was considered to be positive when it was maintained through four successive transfers on the same type of medium and to be negative if no growth occurred over a period of one month. In the course of experiments it was found that media showing an abundant growth in the first transfer in 24–48 hours maintained a rich growth through the four transfers. Media showing scanty and slow growth after the first and second transfer usually did not maintain the growth any further.

The final positive cultures were tested for purity by stroking agar plates or slopes and staining smears. The stock agar cultures from which the transfers of *B. solanacearum* and *A. michiganense* have been made, were finally tested for pathogenicity by reinoculations into the host plants.

## RESULTS.

### TABLE 1.

*Food Requirements of* Bacterium solanacearum.

—— = no growth. + = abundant growth.

| Source of C. | Source of N. | | | | |
|---|---|---|---|---|---|
| | Ammonia. | Asparagine. | Potassium nitrate. | Potassium nitrite. | None. |
| Asparagine | | | | | |
| Glucose | + | + | + | —— | —— |
| Laevulose | —— | + | —— | —— | —— |
| Sucrose | + | + | + | —— | —— |
| Maltose | —— | + | —— | —— | —— |
| Glycerol | + | + | + | —— | —— |
| Sodium acetate | —— | + | —— | —— | —— |
| „ oxalate | —— | + | —— | —— | —— |
| „ citrate | + | + | + | —— | —— |

| Source of N. | Source of C. | | |
|---|---|---|---|
| | Glycerol. | Sucrose. | No Addition. |
| Peptone | + | + | + |
| Tyrosine | + | + | + |
| Glutamic Acid | + | + | + |

Table 1 shows the growth of *B. solanacearum* in synthetic media, containing different sources of carbon and nitrogen.

It is to be observed that asparagine, peptone, tyrosine and glutamic acid serve both as a source of carbon and of nitrogen and maintain the growth of *B. solanacearum*.

Both ammonia and nitrate ($KNO_3$) were shown to be suitable sources of nitrogen, whilst nitrite ($KNO_2$) was not utilized. Of substances providing a source of carbon, glucose, sucrose, glycerol and sodium citrate always produced a rich growth in 24–48 hours in each of the four transfers.

With laevulose, maltose, sodium acetate and sodium oxalate, no growth occurred in transfer media, but sometimes a large inoculum produced slow growth. It was also shown that quick successive transfers of *B. solanacearum* from one agar slope to another produced an invigorated culture and inoculations into the above media gave a quicker and more abundant growth. None of these media,

however, could be considered suitable sources of carbon supply as they did not constantly produce growth through four transfers. When the carbohydrates, sodium salts or glycerol were the only sources of carbon and no source of nitrogen was supplied, a faint growth was sometimes observed in the first and sometimes also in the second transfer.

Comparing the above results with these obtained by Honing, who worked on strains of *B. solanacearum* isolated from tobacco in Sumatra, it may be noted that Honing also observed the variability of the organism in the utilization of compounds. There are, however, points of difference. The strains of the organism he used often failed to show growth in the asparagine medium, while the strain used by the author constantly maintained good growth in this medium. Honing also found nitrites to be a suitable source of nitrogen, while Table 1 shows that nitrites were not utilized by the Victorian strain of *B. solanacearum*.

TABLE 2.

*Food Requirements of* Aplanobacter michiganense.

\+ = abundant growth. —— = no growth.

| Source of C. | Source of N. | | | | |
|---|---|---|---|---|---|
| | Ammonia. | Asparagine. | Potassium nitrate. | Potassium nitrite. | None. |
| Asparagine | —— | | | | |
| Glucose | —— | —— | —— | —— | —— |
| Laevulose | —— | —— | —— | —— | —— |
| Sucrose | —— | —— | —— | —— | —— |
| Maltose | —— | —— | —— | —— | —— |
| Glycerol | —— | —— | —— | —— | —— |
| Sodium acetate | —— | —— | —— | —— | —— |
| „ oxalate | —— | —— | —— | —— | —— |
| „ citrate | —— | —— | —— | —— | —— |

| Source of N. | Source of C. | | |
|---|---|---|---|
| | Glycerol. | Sucrose. | No Addition. |
| Peptone | + | + | + |
| Tyrosine | —— | —— | —— |
| Glutamic Acid | —— | —— | —— |

Table 2 indicates that of the four substances containing both carbon and nitrogen only peptone was found to produce growth. All other combinations of compounds supplying carbon or nitrogen were not suitable for growth. Growth occurred sometimes in the first transfer when a large inoculum was used and sometimes a small amount in the second, but no growth took place in further transfers. The above data confirm Stapp's observations on the utilization of nitrogen compounds by *A. michiganense*, viz. that only some proteins and amino-acids can serve as a source of nitrogen for the organism.

*Morphology.*

In the course of experiments with *B. solanacearum* considerable variation in morphology was noticed and the results of observations are given below.

Fresh isolations from the host plants on to an agar plate showed Gram negative small cocco-bacilli about $0{\cdot}3\mu \times 0{\cdot}4$–$0{\cdot}8\mu$ in size, frequently occurring in pairs. On transferring a small inoculum from one plate to another a poor growth was produced. Morphologically the organism changed into longer and thicker rods with rounded ends about $0{\cdot}5\mu \times 1{\cdot}0\mu$ in size, paired together and often giving the appearance of a large rod with a constriction in the middle. On transferring into broth the cocco-bacillus type again developed. Transfer of the organism from liquid media to agar slopes often caused the appearance of long slender filaments consisting of 10 to 20 cells, each $0{\cdot}5\mu \times 1{\cdot}2\mu$ in size. This filament formation was not recorded by Smith in his studies of the organism, but is mentioned by Honing (loc. cit.).

*A. michiganense* showed a little variation in morphology; it is Gram positive and occurs as single rods about $0{\cdot}4\mu \times 1{\cdot}0\mu$ in size.

*Cultural Characters.*

Certain differences in cultural characters from Smith's (1914, 1920) type cultures of the organisms were observed. It is possible that these are differences in strain and for the complete id ntification of the organisms isolated in Victoria the following table is set out.

TABLE 3.

*Cultural Characters of* B. solanacearum *and* A. michiganense.

——— = no acid, no gas. A = acid. a = faint acid.

| Medium. | *B. solanacearum.* | *A. michiganense.* |
|---|---|---|
| Agar | Greyish white, moist, shiny growth, older cultures become brown. | Yellow, moist, shiny growth. |
| Gelatin | Liquefied slowly after 3 weeks. | Liquefied slowly after $2\frac{1}{2}$ weeks. |
| Broth | Uniform turbidity, later greyish deposit. | Uniform turbidity, later yellow deposit. |
| Litmus milk | Turns blue, sediment. | Turns red, later discoloured, deposit. |
| Lactose | ——— | ——— |
| Glucose | A | a |
| Mannite | ——— | ——— |
| Saccharose | ——— | a |
| Galactose | a | ——— |
| Maltose | ——— | ——— |
| Dulcite | ——— | ——— |
| Salicin | ——— | a |
| Indole formation | Indole not formed. | Indole not formed. |
| Nitrate reduction | Nitrates reduced to nitrites. | Nitrates not reduced to nitrites. |
| $NH_3$ production | Ammonia produced. | Ammonia not produced. |

It is to be observed that *B. solanacearum* produced acid in glucose, while in galactose only a slight amount of acid was formed after some weeks of incubation. The strains of the organism described by Smith failed to produce acid or gas in any of the carbohydrates tested by him. Grieve (1935) recorded acid formation by the Victorian strain of *B. solanacearum* in glucose, but no change in galactose was observed. No pellicle formation was found in broth; this is contrary to the finding of Smith, but was recorded by Grieve.

The *A. michiganense* strain used in this work produced yellow colonies on agar slope; Bryan (1932) recorded pink and white variants. The organism produced slight acid after a long period of incubation in glucose, saccharose and salicin. Smith did not record the formation of acid from carbohydrates, but Bryan (1930) noted acid production from certain carbohydrates.

*Virulence of B. solanacearum.*

Four months after the isolation of *B. solanacearum* the culture was reinoculated into two tomato plants. After one week the plants showed petiole epinasty, and after two weeks the plants showed characteristic wilting of the leaves. Transverse sections of the stem showed xylem invasion. This result is interesting because it shows that under certain conditions *B. solanacearum* may retain its virulence for a considerable period. It has been the experience of most workers, including those in this laboratory, that normally the organism rapidly loses its pathogenicity in culture media. It is suggested, tentatively, that the retention of pathogenicity over a four-month period is due to the rapid subculturing practised during the course of the experiments.

## DISCUSSION.

No reason is apparent why *B. solanacearum* should be restricted to the xylem. Here, although it may obtain inorganic salts (Clements, 1930; Mason, Maskell and Phillis, 1936; Eckerson, 1924), and sugars (Priestley, 1924; Anderssen, 1929), its cultural performance suggests that it might grow equally well in other tissues of the plant, e.g. phloem, which contains simple amino-acids.

*A. michiganense*, which grew in culture only on peptone, might be expected in the phloem as well as other tissues containing peptone.

## SUMMARY.

A study has been made of the carbon and nitrogen food requirements of a xylem invader, *B. solanacearum*, and a phloem invader, *A. michiganense*. It has been shown that the Victorian strain of *B. solanacearum* used in the experiments utilizes asparagine, tyrosine, peptone and glutamic acid both as a carbon and nitrogen source; glucose, sucrose, glycerol and sodium citrate serve as a source of carbon, while ammonia salts and nitrates can supply nitrogen. *A. michiganense*

utilizes only peptone out of all the compounds supplied in the experiments. An attempt has been made to correlate food requirements with solutes present in the vascular tissues.

A study of morphology, cultural characters and virulence of the organisms showed certain variations in the Victorian strains from the *B. solanacearum* E.F.S. and *A. michiganense* E.F.S. types.

## ACKNOWLEDGMENTS.

Thanks are expressed to Dr. Grieve for valuable assistance in the course of this work.

### REFERENCES.

Anderssen, F. G. (1929): Plant Physiol., 4, p. 459.
Bryan, M. K. (1930): Jour. Agric. Research, 41, p. 825.
Bryan, M. K. (1932): Phytopath, 22, p. 787.
Clements, H. F. (1930): Res. Studies, State Coll. Wash., 2, p. 91.
Darnell-Smith, G. P. (1918): Jour. and Proc. Roy. Soc. N.S. Wales, 52, p. 435.
Eckerson, S. H. (1924): Bot. Gazette, 77, p. 377.
Fish, S. and Pugsley, A. T. (1936): Jour. Dept. Agric. Vic., 34, pp. 520 and 528.
Grieve, B. J. (1935): Proc. Roy. Soc. Victoria, 48, p. 79.
Grieve, B. J. (1938): In course of publication.
Honing, J. H. (1912): Mededeel. van Het. Deli Proefst. te Medan, 6, p. 219.
Lewis, T. M. (1930): Phytopath., 20, p. 723.
McAlpine, D. (1911): Potato Diseases in Australia, J. Kemp, Government Printer, Melbourne, p. 87.
Magee, G. J. (1935): Agric. Gaz. N.S. Wales, 46, p. 192
Mason, T. G., Maskell, E. J. and Phillis, E. (1936): Ann. Bot., 50, p. 23.
Priestley, J. H. (1924): New Phytol., 23, p. 161.
Samuel, G (1926): Abst. Rev. Appl. Myc., 5, p. 213.
Smith, E. F. (1896): U.S. Dept. Agric. Div. Veg. Phys. and Path. Bul. 12, p 1.
Smith, E. F. (1910): Science n.s., 31, p. 794.
Smith, E. F. (1914): Bacteria in relation to plant diseases, Carnegie Inst. of Washington, 3, pp. 161 and 174.
Smith, E. F. (1920): An introduction to bacterial diseases of plants, W. B. Sanders Co., Philadelphia and London, pp. 177 and 202.
Stapp, C. (1930): Zeitschr. für Parasitenkunde, p. 756.
Tryon, H. (1894): Ann. Rept. Dept. Agr. Queensland, p. 2.

# TISSUE INJURY BY RADIANT ENERGY AND THE LIBERATION OF HISTAMINE

by

C. H. KELLAWAY, H. F. HOLDEN AND E. R. TRETHEWIE[1]

(From the Walter and Eliza Hall Institute, Melbourne).

*(Submitted for publication 31st October, 1938.)*

When the tissues of living animals are exposed to light, dilatation of capillaries, stasis and emigration of leucocytes result (Finsen, 1893; Dreyer and Jansen, 1905; Campbell and Hill, 1924). For these changes ultra-violet radiations are mainly responsible. The researches of Lewis and his colleagues (Lewis, 1927) pointed to the liberation of a diffusible chemical substance (H-substance) as the cause of the rather slowly appearing triple response in the human skin after exposure to ultra-violet radiation. It seemed therefore worthwhile to ascertain whether histamine could be demonstrated in the perfusate from isolated organs irradiated with ultra-violet light. For this purpose we used the perfused lung of the dog, which is most suitable because a large area can be exposed to radiation and because radiant energy can penetrate to a greater depth in an air-filled than in a solid organ. Since in any case only a small proportion of the cells in the organ could be affected, and only relatively small amounts of histamine could be expected to appear in the perfusate, it was necessary to use a slow rate of perfusion and to make the methods of testing as sensitive as possible.

Under these conditions we have been able to demonstrate a small output of histamine not only after irradiation with ultra-violet but also with visible and infra-red radiations.

## METHODS.

Our experiments were made upon the artificially perfused left lungs of dogs. The dogs weighing 7 to 10 kg. were anaesthetized with chloroform after a preliminary injection of 70 to 110 mg. morphine hydrochloride. The lungs were artificially ventilated and perfused through the pulmonary artery with Tyrode solution at a head of pressure of 80–85 cm. The Tyrode reaching the lung was warmed to 35°C. by passing through a spiral immersed in a large bath at 45°C. Great care was taken to avoid perfusing the lung with fluid at a higher temperature than 36°C. The lung was removed from the body of the dog and placed on a paraffin block arranged at an angle of 45° with the trachea dependent. A special tracheal cannula was used. This had a side piece through which air entered from the pump; the outlet, furnished with a piece of rubber tubing

[1] Junior Research Fellow of the National Health and Medical Research Council. The work was aided by a grant from the National Health and Medical Research Council.

and a screw clamp, regulated the escape of air and allowed the drainage of any fluid which might collect in the bronchi. Ventilation was from 10 to 16 per minute at a stroke volume of 500 c.c. Hyperventilation was sedulously avoided. After the blood had been washed out from the organ by a faster flow of perfusing fluid, the rate was cut down to 2–3 c.c. per minute and remained constant throughout. The general arrangement used in the experiments can be seen in Fig. 1.

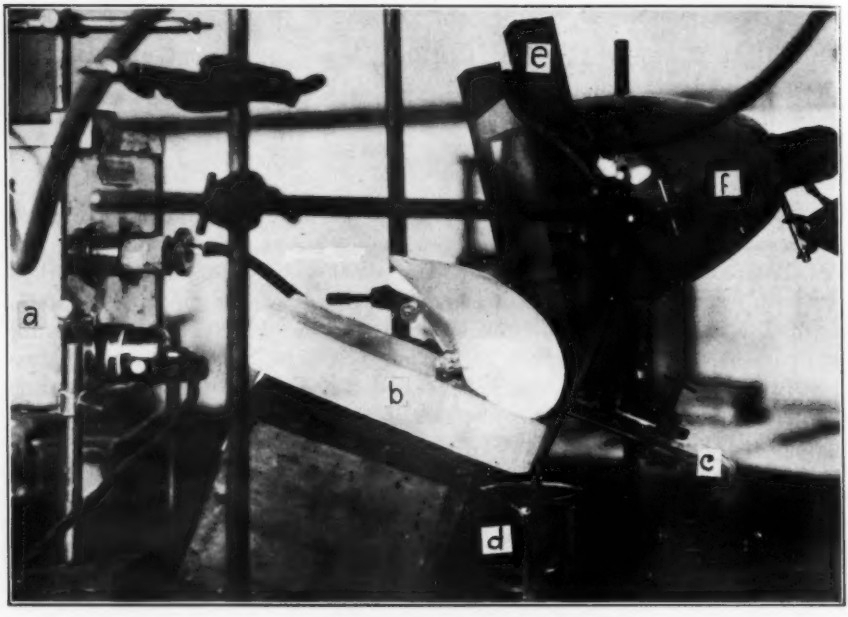



**Fig. 1.** Photograph showing arrangements for irradiation of perfused dog's lung with visible light: a. electrically heated bath containing spiral through which perfusing fluid flows; b. paraffin block on which organ rests; c. tracheal cannula; d. beakers for collecting outflow; e. water screen; f. lamp.

Two light sources were used: a quartz mercury vapour lamp (K.B.B.) and a 60 w. gas filled electric filament globe, the former at a distance of 30 cm. and the latter at 6 cm. (unless stated otherwise) from the surface of the lung. Pure ultra-violet radiation was obtained by screening the quartz mercury vapour lamp with a piece of Wood's glass 1 cm. thick. This was cooled by an air current from a fan so that there was practically no secondary radiation from it to the lung. Pure infra-red was obtained by screening the 60 w. lamp with black photographic paper. It was more difficult to get a source yielding only visible radiation. Radiant heat was eliminated by a trough 2·2 cm. in thickness with glass sides 2 mm. thick made by cementing two plates of glass on to a suitably shaped piece of thick rubber sheet. Through this tap water was circulated from a constant level at a rate sufficient to maintain its temperature 5° or 6° below that of the room (20–22°C.). An air current from a fan cooled the side of the trough next to the lamp. To eliminate ultra-violet radiation from the 60 w. lamp a piece of plate glass 1·2 cm. thick was used

in addition to the trough of water. This was shown to be about as efficient as a layer of corresponding thickness of 3 p.c. quinine solution. Complete elimination of ultra-violet and of all the visible spectrum, except the red and a small part of the orange was obtained by using a piece of photographic ruby glass, 2·0 mm. thick, attached to the front wall of the water trough. The light from these sources and the effects of screening were compared using a spectrograph covering the visible and near ultra-violet range. A rough estimate of the amount of radiant heat reaching the lung was obtained by a delicate thermometer with blackened bulb suspended just in front but not in contact with the lung. The lung was shielded from the air current set up by the fan by means of a cardboard screen and its surface was kept moist by bathing it at regular intervals with the outflowing fluid.

Histamine was estimated on the isolated jejunum of the guinea-pig and expressed as di-hydrochloride. The isolated organ bath had a volume of 5 c.c. and by using test doses of 1·0 c.c. of perfusate, histamine could be estimated in concentrations as low as 1: 50 millions, the gut being sensitive to 0·02$\gamma$ of histamine. In those experiments in which an output up to or in excess of 1: 10 millions was obtained the estimations were checked by assay on the cat's blood pressure before and after the administration of atropine. For the estimation of histamine the samples were brought to the boiling point and immediately cooled. Assay was also made for slow contracting substance on unboiled samples (Feldberg and Kellaway, 1938a). The histamine present in the lung under experiment was estimated by determination of the content of a boiled saline extract of a piece of the perfused right lung since, as Feldberg and Kellaway (1937) have shown, the histamine content of the two lungs in cats and guinea-pigs does not differ by more than 20 p.c.

## RESULTS.

Our results show that all the forms of radiant energy tested cause the liberation of histamine from the dog's lung.

It was first necessary to demonstrate that under the conditions of our experiments, and particularly with the slow rates of perfusion which we used, histamine was not liberated in estimable amounts during four or five hours. We did several such control experiments and found that the lung could be perfused without any significant output of histamine into the perfusate, provided that the ventilation and the head of perfusion pressure were kept constant. No fluid flowed from the trachea, and none collected in the lung except in those parts which were dependent and below the level of the hilum.

Since the output of histamine from perfused organs subjected to any form of tissue injury depends largely upon the content of histamine in the organ, we record the result of such a control experiment with a lung containing from 3–4 times the average amount present in lungs used in our experiments.

A lung weighing 50 gm. and containing 120$\gamma$ histamine per gram was perfused for four hours at 2·0 c.c. per minute, and samples of perfusate were tested at intervals of 15 and 30 minutes. The samples collected during the first 1½ hours contained no detectable histamine; during the next two hours the samples contained amounts too small to be estimable—less than 1 : 50 millions. During the next half-hour the perfusate contained 1 : 50 millions. The preparation was now used for the photograph of Fig. 1, and it was necessary to stop the ventilation and keep the lung distended with air while a number of exposures was made. The rate of flow

during this half hour was only 1·0 c.c. per minute, and the histamine content of the perfusate rose to 1 : 20 millions, the output in γ per hour being but little increased. The sample in the final half-hour also showed decreased rate of flow, and the concentration of histamine in it was 1 : 17 millions. The lung was now injected with dye (eosin) and was shown to be satisfactorily perfused in every part; when cut down and drained it yielded 34 c.c. of fluid in 30 minutes. At no time during the experiment was there any flow of fluid from the trachea. Part of the fluid draining from the lung when it was cut down therefore came from the dependent parts of the lung below the level of the hilum, and part from the vessels of the organ.

When lungs perfused in this manner are injected with snake venom there is a rapid onset of oedema and a copious outflow of tracheal fluid rich in histamine. This output is clearly the result of the tissue-injuring actions of the venom. In only three of the experiments recorded here was there any outflow of tracheal fluid during their course, and in two of these it was trivial. When the lung was cut down varying amounts of drainage fluid were collected. We have estimated the histamine in this fluid but have not graphed it in our figures, since the liberation of the histamine found in it cannot be ascribed with certainty to the action of radiant energy upon the lung.

(a) *Unscreened Mercury Vapour Lamp.*

The effect of the unscreened radiations from a quartz mercury vapour lamp on the isolated perfused lung of the dog was an immediate liberation of histamine into the venous perfusate. In the experiment of Fig. 2 the dog's left lung, weighing 58 gm. and containing 45γ histamine per gm., was exposed for one hour to radiation from this source. During this period 22γ of histamine, and during the following two hours 13γ and 7γ, were estimated in the perfusate. At the end of the second hour after exposure 1·4 c.c. of tracheal fluid was obtained containing 1 : 29 millions histamine, and drainage fluid collected during twenty-five minutes amounted to 60 c.c. containing 1 : 5 millions histamine.

In this experiment the lung was exposed to ultra-violet, visible and infra-red radiations. The lamp at the same distance (30 cm.) produced erythema of the skin of the forearm of one of us (C.H.K.) after an exposure of one minute; and exposures for one-and-a-half, three and four minutes produced erythema within one hour. These skin effects were probably produced by radiations of less than 3000Å. A rough estimate of the intensity of the radiant heat from this source was given by the rise of temperature of a thermometer placed in front of the lung from 20° C. (room temperature) to 29° C. during the exposure.

In several experiments of this kind we estimated the histamine content of the lung at the end of the experiment, and found that it did not differ significantly from that of the right lung. This was to be anticipated since the small amount of histamine liberated must have come from a volume of lung equal to the surface

area irradiated by the depth to which the radiations penetrated. This necessarily formed only a small proportion of the lung. In most of our subsequent experiments we did not estimate the histamine remaining in the lung at the end of the experiment. In a few experiments, however, we estimated the histamine in a thin piece of lung cut from the irradiated surface, and found that this showed a fall in

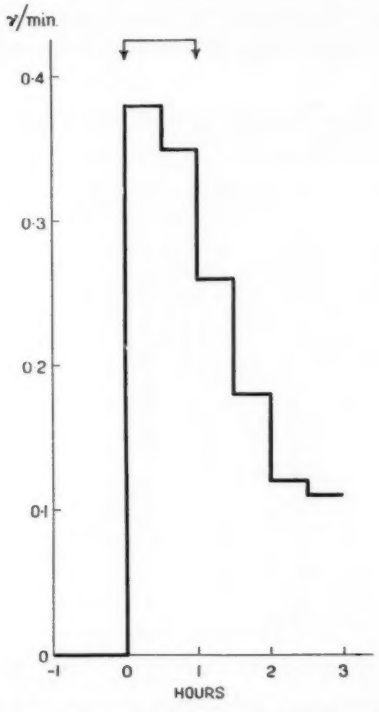



**Fig. 2.** Output of histamine from perfused lung caused by unfiltered light from mercury vapour lamp. Ordinates: output of histamine in γ per minute. Abscissae: time in hours. The arrow heads mark the time during which the lung was irradiated.

histamine content, whereas extracts of unirradiated surface have normally about the same content of histamine as extracts of mixed samples taken from several parts of the organ so as to give an average. For example, in one experiment the weight of the lung before was 47 gm., and the right lung contained 60γ histamine per gram. After the experiment, in which the lung was exposed to unscreened ultra-violet radiation for one hour, it weighed 48 gm. and the histamine content of an average sample was not significantly altered. An extract made from the irradiated surface contained only 50γ histamine per gram. In another experiment the weight of the

left lung before was 31 gm. and after 43 gm., the right lung contained 59γ per gram and an average sample of the left lung after contained 57γ per gram (corrected for oedema). The irradiated surface of the left lung after this exposure to ultra-violet contained only 33γ histamine per gram. It is doubtful whether the histamine content of this part of the lung should be corrected for oedema, most of which was in the dependent parts of the lung below the hilum. The exposed region was well filled with air and contained no obvious collection of fluid. If corrections were made for oedema the histamine content of this region of the lung would still be only 47γ.

(b) *Mercury Vapour Lamp Screened with Wood's Glass.*

Ultra-violet radiation filtered from visible and infra-red radiation by a screen of Wood's glass also caused an immediate but smaller liberation of histamine. In

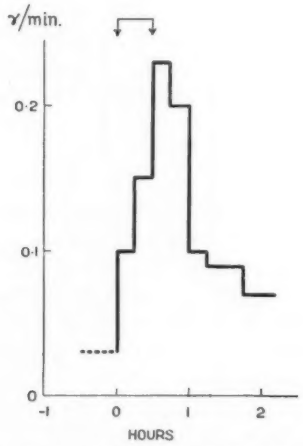



**Fig. 3.** Output of histamine from perfused lung irradiated with light from mercury vapour lamp filtered through a screen of Wood's glass. Ordinates and abscissae as in Fig. 2.

the experiment of Fig. 3 the dog's left lung weighing 43 gm. and containing 60γ of histamine per gm. was exposed to this source for 30 minutes. During the half-hour before exposure 0·9γ, during the period of exposure 3·7γ, and in the three succeeding half-hour periods 6·5γ, 2·9γ and 2·4γ of histamine were estimated in the perfusate. The lung was exposed for a second time to the same light source, 5 mg. of sodium protoporphyrinate being injected. During the half-hour of exposure 5·2γ, and in the succeeding half-hours 6·5γ, 4·4γ and 3·7γ histamine were estimated in the perfusate. The drainage fluid collected in half-an-hour amounted to 32·5 c.c. and contained 1 : 6 millions histamine.

The screen used transmitted light between 3750 and 3300Å. At 3650Å about 1·5 p.c. and at 3350 about 0·3 p.c. of the radiations were transmitted. The temperature of the thermometer placed in front of the lung rose only 1·5° C. above room temperature.

(c) *Gas Filled Electric Filament Globe (Unscreened).*

This source of radiant energy emitting infra-red, visible and some ultra-violet radiations also caused the liberation of histamine from the lung.

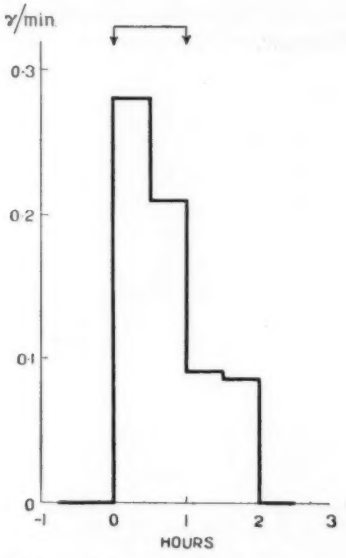



**Fig. 4.** Output of histamine from perfused lung irradiated with unscreened 60 w. filament lamp. Ordinates and abscissae as in Fig. 2.

Fig. 4 shows the histamine output from a lung weighing 50 gm. and containing 40γ histamine per gm. which was exposed for one hour to the 60w. lamp. During this period 15γ of histamine and in the following hour 5γ were estimated in the perfusate. The succeeding half-hour sample contained no demonstrable amount of histamine. There was no outflow of tracheal fluid, and the drainage fluid collected at the end of the experiment amounted only to 5 c.c. containing 1:4·5 millions histamine.

The output of light, both visible and ultra-violet, was very small compared with its intensity from the mercury vapour lamp, being of the order of 1/60th over the range 4000 to 3400Å. No erythema was caused on the skin of the forearm of C.H.K. by 30 minutes' exposure at 6 cm. distance—the skin being kept cool by an

air current. This is probably accounted for by the absence of radiation of less than 3000Å. The thermometer in front of the lung rose from 21° to 26°C and the radiant heat was probably somewhat less than in the experiment of Fig. 2.

(d) *The Effect of Visible Light Alone.*

A number of experiments was made using as a source the 60w. gas-filled lamp. This source could not be freed completely from ultra-violet radiation without the use of a coloured glass screen, but only trivial amounts (about 1 p.c. of that emitted from the quartz mercury lamp screened by Wood's glass) were transmitted in the experiments in which white light was used. In all of them there was a small but significant output of histamine.

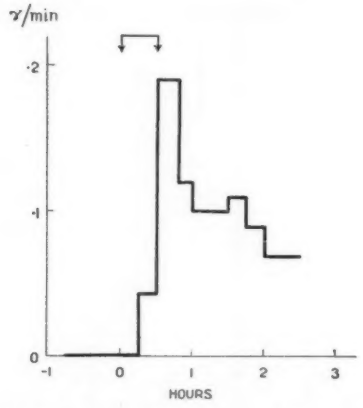



**Fig. 5.** Output of histamine from perfused lung irradiated with a 60 w. lamp screened with 2·2 cm. of water. Ordinates and abscissae as in Fig. 2.

A lung weighing 47 gm. and containing 23γ per gram was exposed for one hour to light from the 60w. lamp screened with the water-trough and 1·2 cm. of plate glass. The output of histamine in the half-hour preceding exposure was nil, during the first half-hour less than 1γ, during the second half-hour 1·6γ and in the first half-hour after exposure 1γ was estimated. The output now was again too small to estimate. A second exposure for one hour to the same source without the plate glass screen also gave only a trivial output of histamine.

In Fig. 5 we record the result of an anomalous experiment in which we obtained, after exposure for half-an-hour to the same light source with the water-filter but without the plate glass screen, a much larger output of histamine, and at the end of two hours after exposure an outflow of tracheal fluid. Since the degree of tissue injury caused by radiant energy in this experiment must have

been small unless the lung was unusually sensitive, we have hesitated to ascribe the output in the drainage fluid at the end of our experiments to the effects of radiant energy. In this experiment the lung weighed 48 gm. and contained 38γ of histamine per gram. During the half-hour of exposure 0·7γ and in the succeeding half-hour periods 5·2γ, 2·8γ, 3·0γ and 2·0γ histamine were estimated in the perfusate. It will be noted that there was some delay before the output began. At the end of the second hour after exposure 16·5 c.c. of tracheal fluid containing 1 : 4 millions histamine and in the next half-hour 14·0 c.e. containing 1 : 10 millions were collected. The drainage fluid collected during 20 minutes amounted to 14 c.c. and contained 1 : 8 millions histamine. The temperature of the thermometer (in front of the lung) did not rise above the temperature of the room.

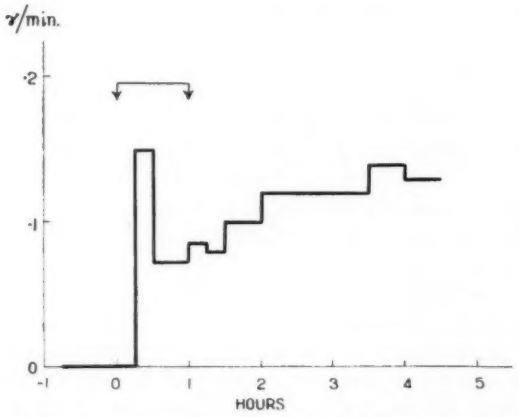



**Fig. 6.** Output of histamine from perfused lung irradiated with red light. Ordinates and abscissae as in Fig. 2.

In the experiments in which a ruby glass screen was used we can certainly exclude the action of any ultra-violet radiation. In the experiment of Fig. 6 a lung weighing 75 gm. and containing 70γ per gm. of histamine was exposed for one hour to the 60w. lamp screened by the water-trough and by a sheet of ruby glass. Before exposure no histamine could be detected in the perfusate. During the hour of exposure 3·7γ and in the succeeding hours 5·5γ, 7·4γ and 7·9γ of histamine were estimated in the perfusate. The drainage fluid in 30 minutes amounted to 9·5 c.c. and contained 1 : 6·7 millions histamine. The temperature measured in front of the lung was only 0·6° C. above room temperature during the exposure, so that no appreciable amount of radiant heat contributed to this output of histamine.

(e) *Infra-red Radiation.*

In Fig. 7 is shown the output of histamine from a lung weighing 37·5 gm. and containing 15γ histamine per gm. which was exposed to the infra-red radiation from a 60w. lamp 4 cm. distant. Visible and ultra-violet light were excluded by a screen of black photographic paper. The temperature in front of the lung (measured on a delicate thermometer with a blackened bulb) rose from 22° to 36° C. During the half-hour before exposure 1·7γ, during the hour of exposure 11·5γ and during the succeeding hours 6·7γ and 5·9γ of histamine were estimated in the perfusate. A small amount of tracheal fluid, 0·4 c.c., was collected during the last half-hour of this experiment, and when the lung was cut down 20 c.c. of drainage fluid were collected in 20 minutes.

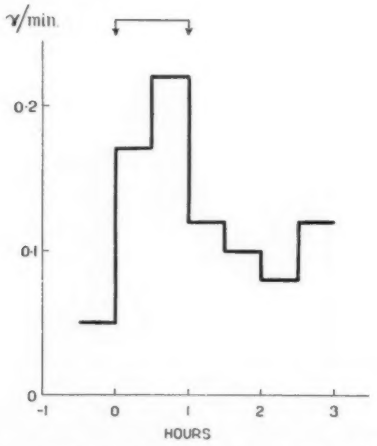



**Fig. 7.** Output of histamine from perfused lung caused by infra-red radiation. Ordinates and abscissae as in Fig. 2.

It will be noticed that this is one of the few experiments in which a small but definite output of histamine was recorded before exposure. Had we perfused the lung a little longer before exposure this would have diminished still further. The amount of radiant heat falling on the lung was greater than in any of our recorded experiments, and in response to this severe stimulus a relatively large output of histamine occurred, but in three hours this only amounted to 4 p.c. of the histamine in the lung.

*Slow Contracting Substance.*

The perfusate collected from these experiments before exposure of the lung to radiant energy seldom contained any significant amount of the slow contracting

substance described by Feldberg and Kellaway (1938a). In none of these experiments was any increase in slow contracting substance detected in the perfusate during or after irradiation, though large doses (1·0 c.c.) of perfusate were used for testing.

In these experiments there appeared to be a slight increase of protein coagulated by boiling in the perfusate during and after irradiation, but the amounts present were too small to be estimated by Esbach's method.

## DISCUSSION.

The absorption of all the forms of radiant energy used—ultra-violet, visible and infra-red—by tissue cells causes injury which results in alteration in permeability and liberation of part of their histamine. Though in these experiments the amounts of histamine estimated in the perfusate are trivial, even in comparison with those in experiments with a relatively weak injurious agent like peptone (Feldberg and O'Connor, 1937), it must be remembered that the injurious agent cannot penetrate far into the perfused lung and that only a very small proportion of the cells in the organ are exposed to its action. We have at least been able to show a small but probably significant loss of histamine from the total store in the surface of the lung exposed to radiation.

Our experiments do not allow us to make a strict quantitative comparison of the effects of the different types of radiation. Ultra-violet appears to be the most, and visible radiation the least, effective in causing injury to the cells. With visible and red light there was a slight lag in the output of histamine.

Ultra-violet of about 3000Å, in view of its action on human skin, might be expected to cause some output of histamine from the perfused lung, and if the radiations, including the visible and infra-red, were allowed to cause a local rise of temperature in the regions where they were absorbed, our results would occasion no surprise. However in the experiments recorded here the surface of the lung was kept moist and, with an air current from a fan causing secondary currents in the vicinity, the temperature of the surface of the organ was kept considerably lower than that of the fluid used to perfuse it. The temperature therefore cannot have risen, even in small areas, above physiological limits, and the changes described cannot be attributed to local heating effects but rather to a directly injurious action of all the wave-lengths studied.

Concerning the nature of the tissue injury caused by radiant energy we can offer no satisfactory explanation. It resembles the effect of coagulative necrosis by mercuric chloride (Feldberg and Kellaway, 1938b) rather than that of the venoms and staphlyococcal toxin, because the histamine appearing in the perfusate is not accompanied by any increase in the amount of slow-contracting substance. It is perhaps significant that in tissue injury caused by venoms, when the dose injected into a perfused organ is too small for any appreciable amount of histamine to be liberated—slow-contracting substance is formed and appears in the

perfusate. Though we can express no opinion as to the severity of the tissue injury caused in the small number of cells affected by radiant energy in our experiments, we may regard it as being of a different nature from the injurious effects of venoms which are mediated by lysocithin and attended by the formation of slow-contracting substance.

## REFERENCES.

Campbell, A. and Hill, L. (1924): Brit. J. exp. Path., 5, p. 317.

Dreyer, G. and Jansen (1905): Mitt. Finsen Med. Lysinstit. 9, p. 180. Cited by Campbell and Hill.

Feldberg, W. and Kellaway, C. H. (1937): J. Physiol., 90, p. 257.

Feldberg, W. and Kellaway, C. H. (1938a): J. Physiol. (In the press.)

Feldberg, W. and Kellaway, C. H. (1938b): Austral. J. exp. Biol., 16, p. 249.

Feldberg, W. and O'Connor, W. J. (1937): J. Physiol., 90, p. 228.

Finsen, N. R. (1893): Hospitalstidende 4, p. 1069. Cited by Campbell and Hill.

Lewis, T. (1927): "The Blood Vessels of the Human Skin and their Responses". Shaw & Sons, London.

# SYNTHESIS OF ACETYCHOLINE BY BRAIN TISSUE

by

E. R. TRETHEWIE[1]

(From the Walter and Eliza Hall Institute, Melbourne).

*(Submitted for publication 11th October, 1938.)*

## INTRODUCTION.

Mann, Tennenbaum and Quastel (1938) have recently brought forward evidence pointing to the synthesis of acetylcholine by brain tissue especially in the presence of glucose. They were not aware, however, of the high content of preformed acetylcholine in the tissue demonstrable by heating or by very thorough grinding (Trethewie, 1938). At Dr. W. Feldberg's suggestion I have repeated some of their experiments and have confirmed the observation that acetylcholine is synthesized by brain tissue of the guinea-pig in the presence of glucose and oxygen.

## METHODS.

The methods used were those described in the earlier communication (1938), except that the brain was ground up in eserinized glucose-free Tyrode solution (1 gm. brain to 10 c.c. extract). The concentration of eserine in the extracts was 1 : 300,000 except for one experiment in which it was 1 : 60,000. The extract was divided into three equal parts. One was brought to the boiling point by heating for 60–70 seconds, cooled and at once tested. The remaining portions were incubated at 37° C. for one or two hours after adding 25 p.c. glucose to one of them to make the solution 0·02 M. For incubation they were placed in a layer 0·8 cm. deep in small beakers which were covered by a watch glass. Extra oxygen was supplied by displacing the air over the extract by a continuous stream delivered through a capillary tube above the surface of the liquid. After incubation the extracts were made up to their original volume to compensate for evaporation. The solutions were now boiled, cooled and tested on the isolated frog rectus together with the first sample which was re-tested as a further control. The extracts were allowed to stand for ten minutes and the supernatant fluid was decanted before being diluted for testing.

[1] This work was carried out under a grant from the National Health and Medical Research Council.

## EXPERIMENTAL.

It was first necessary to ensure that the whole of the preformed acetylcholine present in the tissue was liberated into solution. The increase in acetylcholine yield demonstrated by Corteggiani *et al.* (1936) has been shown to be due to the more complete liberation of preformed acetylcholine already present. Heating Tyrode extracts of guinea-pig brain in this way does not always give as high a value for the acetylcholine content as does bringing the solution to the

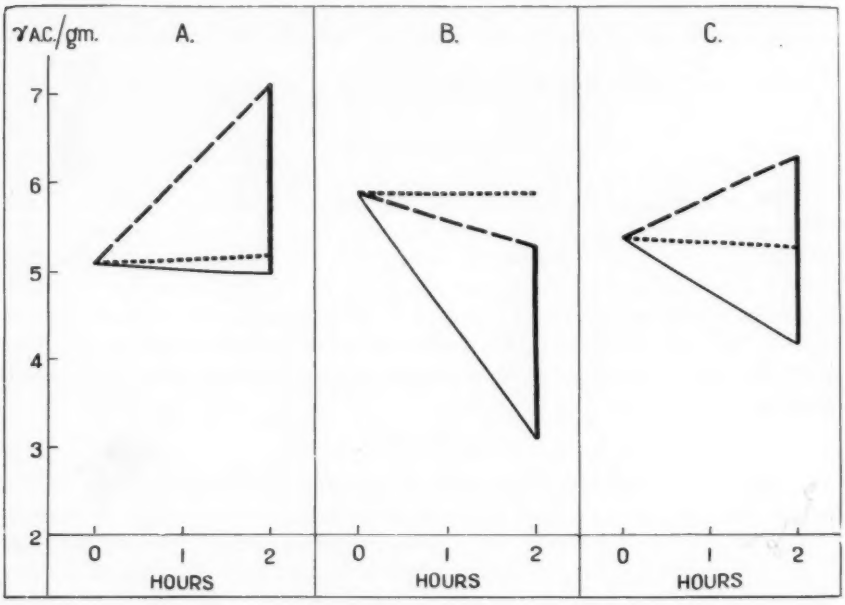



**Fig. 1.** *A.* Incubation with continuous supply oxygen, eserine 1: 300,000.
*B* and *C.* Incubation without extra supply of oxygen, eserine in *B* 1: 300,000
eserine in *C* 1: 60,000.

Added glucose: broken line.
No added glucose: fine continuous line.
Unincubated control: dotted line.
Synthesis shown by heavy vertical line.

boiling point and then immediately cooling. Thus in one experiment the acetylcholine yield after heating to 70°C. was 4·0γ, and after boiling was 4·5γ, an increase of 12·5 p.c. This is attributable to the more complete disruption of the cells by boiling. On the other hand in an experiment where one sample was boiled a value of 5·4γ per gram was obtained, while a second sample boiled twice gave a figure of 5·5γ.

I have carried out six experiments, three in which the incubated samples were freely supplied with oxygen, and three in which no extra oxygen was supplied. In one of the latter a higher concentration of eserine was employed. The experiments gave concordant results. Incubation without oxygen (eserine 1: 300,000) was attended by a fall of acetylcholine which is smaller in the presence of glucose. With eserine 1: 60,000 there was a rise with glucose and a fall without glucose. Incubation with extra oxygen resulted in a rise when glucose was present and a small fall in its absence. In four experiments in which incubation was for two hours the difference between the acetylcholine content in the incubated samples with and without glucose was regularly 40 p.c. In the figure are shown graphically the results in three of these experiments: A, incubation with extra oxygen, B and C without extra oxygen. In C the higher concentration of eserine was employed. In all three cases there was no significant change during the experiment in the acetylcholine per cent. of the un-incubated sample (dotted line). In the samples without added glucose (thin continuous line) the content of acetylcholine fell in A 0·1γ, in B, 2·9γ, and in C, 1·2γ. In the samples with glucose (interrupted line) it rose 2·0γ in A, fell 0·6γ in B, and rose 0·9γ in C. The amount of acetylcholine synthesized per gram tissue is indicated by the heavy vertical line, and was 2·1γ in A, 2·2γ in B and 2·1γ in C.

## DISCUSSION.

In investigations on the synthesis of acetylcholine employing thin slices of brain tissue, Quastel, Tennenbaum and Wheatley (1936) demonstrated an output of acetylcholine into the surrounding fluid under various conditions. More recently Mann, Tennenbaum and Quastel (1938) have brought forward evidence suggesting synthesis by ground-up brain extract of various animals. However, since it has been shown (Trethewie, 1938) that the inactive "precursor" described by Corteggiani *et al.* (1936) is actually only the acetylcholine present in incompletely fragmented cells, and since the liberation of such acetylcholine gives an increase in the acetylcholine yield of four to five times, the evidence brought forward by Quastel, Tennenbaum *et al.* loses much of its force. The evidence of experiments described in this paper in which extra oxygen was supplied demonstrates conclusively the synthesis of acetylcholine in the presence of glucose. Though the highest concentration of eserine used was insufficient to prevent some destruction of acetylcholine, comparison between the effects of two different concentrations of eserine suggests that synthesis occurs in the presence of glucose even without any extra supply of oxygen. This is also indicated by the fact that whether oxygen is supplied or not there is a regular difference of 40 p.c. between incubated samples with and without added glucose. Whether the increase in acetylcholine is attributable to actual formation from glucose or whether it is only a manifestation of increased metabolism of intact cells or of enzyme systems, must for the present be regarded as uncertain.

## CONCLUSION.

Synthesis of acetylcholine in the presence of glucose has been demonstrated for guinea-pig brain.

ACKNOWLEDGMENT.

I wish to thank Dr. C. H. Kellaway for help and encouragement in this research.

REFERENCES.

Corteggiani, E., Gautrelet, J., Kaswin, A. and Mentzer, C. (1936): C.R. Soc. Biol., Paris, 123, p. 667.

Mann, P. J. G., Tennenbaum, M. and Quastel, J. H. (1938): Biochem. J., 32, p. 243.

Quastel, J. H., Tennenbaum, M. and Wheatley, A. H. M (1936): Biochem J., 30, p. 1668.

Trethewie, E. R. (1938): Austral. J. exp. Biol., 16, p. 225

# PHYSIOLOGICAL ONTOGENY IN PLANTS AND ITS RELATION TO NUTRITION

## 5. THE EFFECT OF NITROGEN AND PHOSPHORUS SUPPLIES ON THE RESPIRATION RATE OF THE LEAVES.

by

A. H. K. PETRIE AND R. F. WILLIAMS

(From the Waite Agricultural Research Institute, University of Adelaide[1]).

*(Submitted for publication 5th September, 1938.)*

## INTRODUCTION.

On account of the integral part played by respiration in metabolism, the course of its rate is of considerable interest in the study of the drifts in physiological attributes of plant organs during ontogeny. In two of the experiments described in previous papers of this series—viz. the Sudan grass experiment of paper 1 (Ballard and Petrie, 1936) and Experiment 2 with oats of paper 2 (Williams, 1936)—measurements were made of the respiration rate of the total leaves at the successive harvests. In the present paper these measurements are recorded and determinants sought for the variation in respiration rate with treatment and with time.

### SYMBOLS.

$\mathbf{R}_W$ respiration rate on a dry weight basis.
$\mathbf{R}_P$ respiration rate on a protein-nitrogen basis.
$P$ percentage protein-nitrogen content of the leaves, as estimated by tannic-acid precipitation.

## THE MEASUREMENT OF RESPIRATION RATE.

Respiration was measured on the total leaves of plants whose number was varied from harvest to harvest in order to obtain a rate of carbon-dioxide production that tended to be always of the same order; the number ranged from several hundred at harvest 1 to two or three when the plants were fully grown. At each harvest measurements were made on duplicate samples for each treatment.

The leaves were severed at the ligule at about 11 a.m. on the day of harvest, and placed in respiration chambers of 500 ml. capacity maintained at a temperature of 10°C.[2] Air, at this

[1] This represents one of a series of investigations carried out at the Waite Institute and financed co-operatively by the Carnegie Corporation of New York, the Council for Scientific and Industrial Research, and the University of Adelaide.

[2] This temperature was chosen in the hope that values might be obtained of the respiratory index; as will be made clear later in the paper, the $\mathbf{R}_W$ values did not represent this attribute.

temperature and freed from carbon dioxide, was drawn through the chambers at a rate of 11 litres per hour, a stabilization period of at least an hour being allowed before measurements were commenced. It was ascertained that under the experimental conditions the water content of leaves did not fall by more than 1 p.c. The carbon dioxide produced during the periods of measurement was absorbed in Reisset towers and determined by back titration. Three successive measurements were made for each sample, the period for each measurement usually being two hours.

It was found that the three values progressively decreased, except at the last harvest of the Sudan grass experiment, where the first and second values were almost identical and the third lower. Values for $\mathbf{R}_W$ are recorded in Table 1: they are means of values obtained by interpolation on the drift curves at 3 hours after cutting; this procedure was adopted as the drift curves did not permit accurate extrapolation to zero time. To determine the relative rate of fall with time as affected by harvest and treatment, further interpolations were made at 6 hours after the time of cutting. The values so obtained in the Sudan grass experiment are expressed in fig. 4 as a percentage of the respective 3-hour values: in the oats experiment the relative drift values were very variable and, as an analysis of variance revealed no significant effects of time or treatment, they have not been presented; the average fall over the second 3-hour period was, however, almost 20 p.c.

## DESCRIPTION OF ONTOGENETIC DRIFTS AND TREATMENT EFFECTS.

### *The Present Data.*

The differential treatments applied in the two experiments were as follows:

**Sudan Grass**

| | |
|---|---|
| I | 0·129 gm. N per pot. |
| II | 0·371 " " " " |
| III | 0·742 " " " " |

**Oats**

| | |
|---|---|
| I | 0·0080 gm. P per pot. |
| II | 0·090 " " " " |
| III | 0·60 " " " " |

The experimental details are given in papers 1 and 2.

The values for $\mathbf{R}_W$ of Table 1 are given graphically in fig. 1. The table also contains results of analyses of variance. Owing to the means of the pairs of observations of $\mathbf{R}_W$ being correlated with their variance, the analyses were performed on logarithms of the data, which were first multiplied by 10 to avoid negative values (cf. Statistical Appendix to paper 1).

In the case of the Sudan grass experiment it is seen that the drift of $\mathbf{R}_W$ bears certain resemblances to that of the relative growth rate depicted in fig. 8 of paper 1, and to those of the protein, soluble-nitrogen and water contents depicted in fig. 2 of paper 3 (Petrie, 1937): there is an early maximum, lying between harvests 1 and 3, followed by a persistent fall, the relative rate of fall tending towards constancy; no early depression with treatment is indicated, although in other ways the treatment effects tend to be similar in the different variables. As interaction could not be shown to be significant, it is impossible to use the results of the analysis of variance to examine the differences between individual points on the graph; but each successive treatment has significantly raised the mean respiration rate.

In the case of the oats experiment it can be seen, by comparison with fig. 4 of paper 2 and fig. 2 of paper 4 (Williams, 1938), that the curves of $\mathbf{R}_W$, apart from a very high initial point, resemble those of relative growth rate, unit leaf rate, water content and protein-nitrogen content, the similarity of treatment effect on $\mathbf{R}_W$ and $P$ being especially marked. It will later be suggested that respiration may have been limited in the early stages of growth by the concentration of substrates, but it is possible that the high value of $\mathbf{R}_W$ for harvest 1 was the result of carbohydrate being supplied from the endosperm.

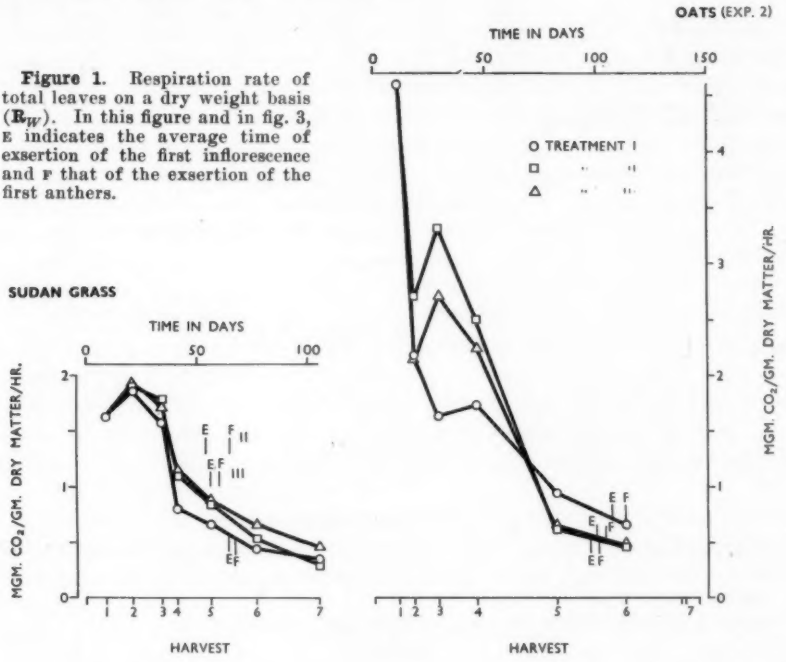



**Figure 1.** Respiration rate of total leaves on a dry weight basis ($\mathbf{R}_W$). In this figure and in fig. 3, E indicates the average time of exsertion of the first inflorescence and F that of the exsertion of the first anthers.

Results of the analysis of variance are given in Table 1. In respect to the values with treatment II, significant depressions in $\mathbf{R}_W$ occur with treatment I at harvests 2–4, and with treatment III at harvests 2–3. At each of harvests 5 and 6 the values with treatment I are significantly greater than those with II and III. In general there is a marked fall with time in $\mathbf{R}_W$ with all treatments.

*Results of Other Investigations.*

Certain work has already been recorded on the effect of nitrogen and phosphorus supply and of age on respiration rate, the results of which in general agree

TABLE 1.

*Respiration Rates of the Leaves.*

SUDAN GRASS.

| Harvest | $R_W$ Treatment I | $R_W$ Treatment II | $R_W$ Treatment III | $R_P$ Treatment I | $R_P$ Treatment II | $R_P$ Treatment III |
|---|---|---|---|---|---|---|
| 1 | 1·62 | 1·62 | 1·62 | 47·0 | 47·0 | 47·0 |
| 2 | 1·86 | 1·90 | 1·93 | 48·4 | 47·6 | 49·9 |
| 3 | 1·57 | 1·79 | 1·72 | 56·9 | 56·1 | 52·4 |
| 4 | 0·80 | 1·11 | 1·15 | 36·4 | 37·2 | 37·5 |
| 5 | 0·67 | 0·84 | 0·88 | 66·3 | 50·9 | 37·1 |
| 6 | 0·44 | 0·53 | 0·66 | 66·3 | 43·4 | 38·6 |
| 7 | 0·34 | 0·29 | 0·47 | 102 | 44·2 | 41·2 |
| Treatment means of log $10R_W$ for hvsts. 2-7. | *0·893* | *0·946* | *1·000* | | | |

ANALYSIS OF VARIANCE OF log $10R_W$.

Analysis performed on data of harvests 2-7.
Interaction insignificant.

S.E. for treatment means (means of 12 observations) = *0·0148*.

OATS.

| Harvest | $R_W$ Treatment I | | $R_W$ Treatment II | | $R_W$ Treatment III | | $R_P$ Treatment I | $R_P$ Treatment II | $R_P$ Treatment III |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 4·60 | | 4·60 | | 4·60 | | 108 | 108 | 108 |
| 2 | 2·18 | *1·337* | 2·71 | *1·431* | 2·15 | *1·331* | 45·3 | 53·5 | 47·2 |
| 3 | 1·63 | *1·211* | 3·31 | *1·520* | 2·71 | *1·431* | 40·1 | 64·1 | 57·7 |
| 4 | 1·73 | *1·236* | 2·51 | *1·399* | 2·25 | *1·351* | 47·7 | 56·5 | 53·4 |
| 5 | 0·94 | *0·972* | 0·62 | *0·792* | 0·65 | *0·813* | 46·3 | 76·6 | 97·0 |
| 6 | 0·66 | *0·820* | 0·46 | *0·663* | 0·47 | *0·667* | 43·4 | 112 | 97·9 |
| 7* | — | | — | | — | | | | |

ANALYSIS OF VARIANCE OF log $10R_W$.

Analysis performed on data of harvests 2-6. The means of the pairs of values of log $10R_W$ are given in italic figures.

Interaction of time and treatment significant.

S.E. for individual values (means of 2 observations) = *0·023*.

SYMBOLS.

$R_W$ respiration expressed in mgm. $CO_2$ per gm. dry weight of total leaves per hour.

$R_P$ respiration expressed in mgm. $CO_2$ per gm. protein nitrogen in total leaves per hour; calculated from the values of $R_W$ given in this table and the values of $P$ given in papers 3 and 4.

* At harvest 7 the leaves were dead.

with those of the present paper. Brief reference to this work will be made before proceeding with the present discussion.

Kidd, West and Briggs (1921) measured the drift in respiratory index of the leaves of *Helianthus* during the growing period. This quantity is the rate of respiration when the amount of respiring material is in excess, the internal concentration of oxygen is non-limiting, and the temperature is 10° C. They regarded

$R_W$ under these conditions as a measure of the effective amount of respiring cell-matter per gramme dry weight, which they termed the "internal factor" in respiration. They found that the respiratory index of the total leaves underwent a continuous decline during the growing period, and concluded that this was an expression of the fact that the respiratory index of individual leaves also decreases with time. They also observed a close resemblance between the drifts of respiratory index and relative growth rate. Similar drifts were obtained for respiration measured at higher temperatures by Inamdar, Singh, and Pande (1925) and Singh (1928).

Gregory and Richards (1929) measured $R_W$ at 25° C. in successive leaves of barley at the time of complete emergence; nitrogen-deficient plants had lower rates than fully manured, but phosphorus deficiency produced no significant effect. Later work (Richards, 1938) showed that more marked phosphorus deficiency caused a progressive decrease in respiration rate. Gregory and Sen (1937) made similar measurements, and repeated them at intervals over a short period following complete emergence; in general they found a decline with time, and again $R_W$ was greatly reduced by nitrogen deficiency.

Hamner (1936) found in tomato and wheat plants that increasing nitrogen supply increased the respiration rate of leaves attached to the plant.

Lyon (1924) found that increasing phosphorus supply to *Elodea* increased the respiration rate, and Jones (1936) found that phosphorus deficiency in wheat seedlings reduced the rate.

Chatterjee (1933) injected leaves with phosphate solutions and found that, provided carbohydrates were abundant, there was an immediate increase in respiration rate. Increased respiration rate of leaves on feeding with phosphorus is also reported by Gregory (1937).

Smirnow (1928) recorded respiration drifts in the leaves of tobacco and sunflower plants, but the method of sampling is not described with sufficient clarity to enable his data to be considered in the present connection.

## DISCUSSION.

### *The Relation of Respiration Rate to the Protein Content.*

Since, as pointed out in paper 1, relative growth rate has not a direct physiological significance, the same can be said for the similarity in the drifts of relative growth rate and $R_W$ noticed by various workers. The similarity must be due to the fact that respiration on the one hand, and metabolic processes determining the leaf weight ratio and unit leaf rate on the other, tend to be affected in like manner by treatment and undergo related ontogenetic drifts. The similarity in behaviour extends to other quantities also, e.g. percentage protein-nitrogen and water in the leaves, and to a less extent to percentage soluble-nitrogen therein. An important factor determining the general decline in these attributes of the plant must be the

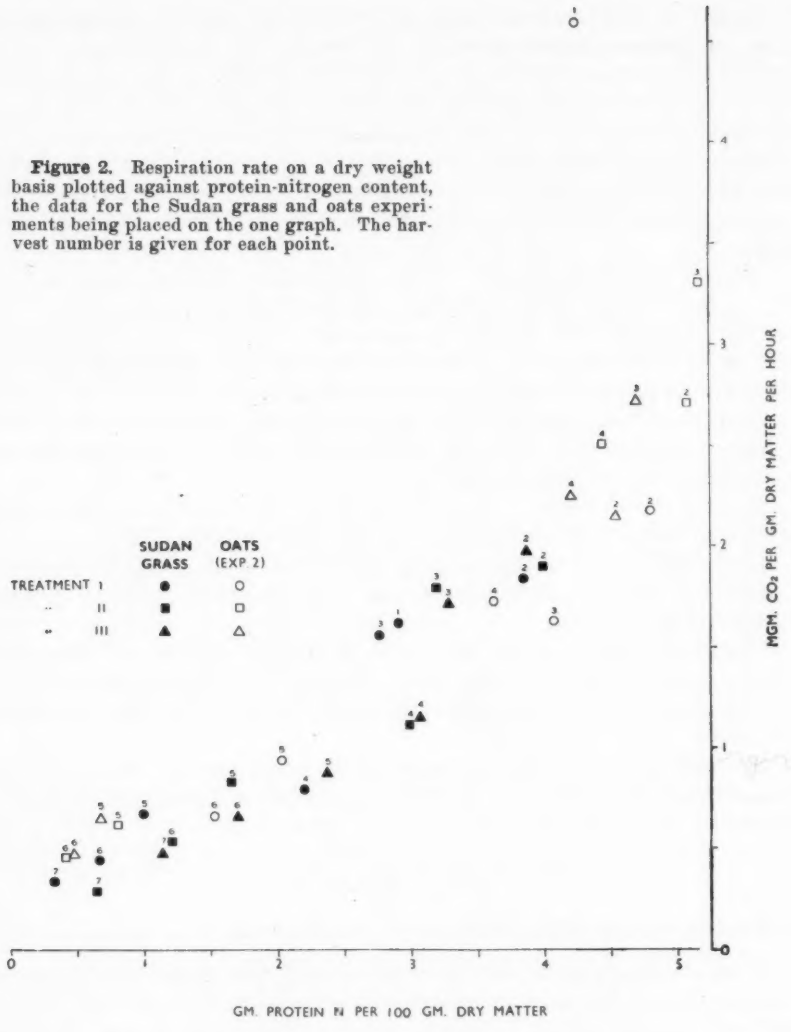



**Figure 2.** Respiration rate on a dry weight basis plotted against protein-nitrogen content, the data for the Sudan grass and oats experiments being placed on the one graph. The harvest number is given for each point.

progressive differentiation of the leaves, and the increasing proportion of non-living material that they contain; this has already been suggested by Kidd, West and Briggs (1921) and Gregory and Richards (1929). Differentiation may also be a factor in the treatment effects: greater thickness of cell-wall has been observed visually in leaves of nitrogen-deficient plants by Mothes (1932) and Suzuki (1935).

A large and varying proportion of the dry matter of the plant is composed of non-living cell-walls and reserve carbohydrates, and the amount of dry matter is thus no measure of the magnitude of the seats at which metabolic processes occur. We have no exact measure of the amount of cytoplasm in the leaves, but it is probable that a rough one will be given by the amount of protein present; and it is proposed in this paper to discuss how accurate this measure may be, and to examine the consequences of assuming it to be a reliable one. In fig. 2 $\mathbf{R}_W$ has therefore been plotted against $P$, the points for the two experiments being placed on the one graph. A striking relationship is revealed. The graph abstracts from time, and draws attention to a broad general relationship between the two variables rather than to effects caused by other factors. Since there is reason to believe that, in an ontogenetic series of data such as that we are studying, the cytoplasm content of the leaves will be a major determinant of $\mathbf{R}_W$, the relation is what might be expected if $P$ were a general measure of this content.

Sudden changes of protein content induced by the environment would not necessarily be expected from this general relationship to produce correlated changes in $\mathbf{R}_W$; and Petrie and Wood (1938) and Wood and Petrie (1938), studying plants of approximately uniform ontogenetic stage in which differences in protein content had been rapidly induced, actually found no relation of $\mathbf{R}_W$ to $P$. Their experiments suggest that the total leaf protein is not a measure of the seat of respiration: probably hydrolysis of a considerable amount does not destroy any of the seat, although in a general sense $\mathbf{R}_W$, $P$, and the cytoplasm content drift in magnitude in the same way during ontogeny.

In the work already referred to, Gregory and Sen (1937) and Richards (1938) found significant correlations between $\mathbf{R}_W$ and $P$. In certain instances the main correlation was between $\mathbf{R}_W$ and the amino-nitrogen content, the $\mathbf{R}_W$-$P$ correlation resulting from correlation between amino- and protein-nitrogen contents; there were, however, cases in which $\mathbf{R}_W$ was correlated with $P$ and not with amino-nitrogen content. Now leaves of different level differ structurally (cf. Zalenski, 1902–23), and much of the $\mathbf{R}_W$-$P$ correlation may be due to variations in cytoplasm content: Gregory and Sen and Richards, nevertheless, believe there is a more fundamental relation between respiration and protein synthesis, as will be discussed on a later page.

Palladin (1896, 1918), from experiments with wheat seedlings germinated in darkness, found that, so long as carbohydrate supply was adequate, $\mathbf{R}_W$ was proportional to the amount of protein not digested by gastric juice, which he took as a rough measure of the amount of protoplasmic proteins. The variations in his quantities were again of an ontogenetic nature.

The examination of the data so far suggests that, over a range of observations on material differing widely in ontogenetic state, and in which therefore the cytoplasm content of the leaves must be a major factor determining $\mathbf{R}_W$, $P$ is probably a rough index of the magnitude of this factor.

*The Validity of the Protein Content as a Measure of the Content of Cytoplasm.*

There are, as can be seen from fig. 2, deviations from the general relation of $\mathbf{R}_W$ to $P$, and in proceeding to a discussion of these we have to consider further the validity of taking $P$ as a measure of the cytoplasm content of the leaves, or, more specifically, of the seat of respiration[3]; it has already been stated that rapid changes in $P$ may not produce related changes in $\mathbf{R}_W$. We therefore propose to examine the information available as to the nature of the material measured as total protein content.

The nucleoproteins must contribute a discrete fraction to the total protein nitrogen, but the magnitude of this fraction is unknown. If the nucleoproteins were confined to the nucleus the fraction might be small, but it is not yet clearly established that they are absent from the cytoplasm.

There arises next the question of the occurrence in the leaves of storage or paraplasmatic proteins, taking no part in metabolism until hydrolysed. Such proteins exist in seeds and tubers, but their occurrence in leaves is uncertain, although some writers (e.g. Nightingale et al., 1928) have postulated it to explain their data. There is, however, evidence that there is more than one protein in leaves, and it is perhaps relevant to consider this here.

Chibnall (1923, 1924, 1933) claims that a small quantity of a soluble protein occurs in the vacuoles, but it is possible that this may have been present in the cytoplasm (cf. Phillis and Mason, 1937, and Kiesel et al., 1934); in any case he found it to amount to only 1–2 p.c. of the total leaf protein. Chibnall (1926) and Chibnall and Grover (1926) found that there were two proteins in the cytoplasm, one water soluble and the other combined with some substance soluble in alcohol. Thomas (1927) has separated water-soluble and water-insoluble proteins in apple leaves, and gives evidence that there may be only one of the latter; the water-soluble protein was less than 10 p.c. of the total nitrogen of the leaves. Kiesel et al. (1934) found in sugar beet leaves a water-soluble and an alkali-soluble protein, the former present in very small amount. The same two types of protein were found in red beet, and *Citrullus colocynthoides*,[4] a melon. Davies (1926) found two proteins in lucerne, one soluble in sodium carbonate, the other in alkali; he also found two similar fractions in vetch. In other cases (e.g. Davies, 1926; Kiesel et al., 1934; Miller, 1935, etc.) where only one protein fraction has been separated, it is not proved that this represents the total leaf protein.

Foreman (1938) has separated two protein complexes from perennial rye-grass; one of these, he considers, probably represents storage protein in which the

[3] That respiration is localized at specific centres in the cell was suggested long ago by Loeb (1895). More recently Joyet-Lavergne (1928, 1929, 1937) and Giroud (1928) claim to have demonstrated that glutathione is localized in the mitochondria, which, as is shown by staining reactions, have a very low $r$H. Giroud (1929) expressed the belief that the sulphydryl group in the mitochondria is linked to complex protein molecules. Joyet-Lavergne (1932) has also shown that leuco-derivatives of various substances are oxidized in the mitochondrial region, and concludes (1929, etc.) that mitochondria are the sites of respiration in the cell. The matter is also discussed by Needham (1927).

[4] Doubtless an error for *C. Colocynthis* Schrad.

protein is loosely combined with potassium and sodium phosphates; the other includes nucleoproteins and probably calcium or magnesium phosphate combined with protein.

In connection with this evidence that more than one protein may occur in the leaf, it is of interest to consider some work on animal proteins by Block (1934). This investigator finds that the total protein separated from a tissue or from blood serum has a constant and characteristic molecular ratio of arginine, histidine and lysine. He concludes therefore that serum and the tissues examined each contain a single protein complex possessing a characteristic *Anlage* of these basic amino-acids, and that the complex is broken up into the commonly isolated proteins, such as albumin and globulin from serum, by physicochemical treatment.

It is possible that a similar state of affairs may exist in leaves, and that therefore the proteins separated by various solvents are not entities in the cytoplasm, but artificially separated parts of a single complex. Furthermore, even if different proteins occur, there is no evidence as yet that they are not all active components of the cytoplasm. Chibnall and Grover (1926) left cut leaves of *Phaseolus multiflorus* exposed to diffuse daylight for four days with their petioles in water. Under these conditions considerable degradation of cytoplasmic proteins occurred, with subsequent transformation of the products of hydrolysis into asparagin; but the ratio of the soluble to the combined proteins showed no great variation. This would be in agreement with Block's hypothesis, and also fails to show that one of the proteins is functionally different from the others.

Smirnow and Izwoschikow (1930), however, make the assumption that in the starved leaf only reserve proteins undergo hydrolysis, and that the stable plasma-proteins remain. They found that, in preliminary air-drying of tobacco leaves, there was considerable protein loss; during subsequent fermentation there was no loss, because then only the stable plasma-proteins remained. They found that in young leaves a greater proportion of the protein was hydrolyzed during starvation than in old ones, and suggested that young leaves contain a greater proportion of reserve protein. They also investigated the composition of the insoluble nitrogen during the course of the starvation. They consider that the composition changed, but this conclusion depends on one increase in basic nitrogen from 19 to 22 p.c. of the total insoluble nitrogen, and therefore seems hardly justified. Their data actually suggest that there was no appreciable change in the proportions of basic, amino- or ammonia-nitrogen during the course of starvation.

It must thus be regarded as still uncertain whether storage proteins exist in leaves chemically distinct from those forming the structural basis of the cytoplasm.

*Respiration Rate on a Protein Basis.*

There remains then some doubt as to the accuracy with which $P$ measures the cytoplasm content of the leaves. Nevertheless it seems likely that the two quantities pass through gross changes in the same direction during ontogeny, so that, in considering ontogenetic drifts, protein content may be a better basis upon which

to express respiration rate than dry weight; furthermore in view of the observed $\mathbf{R}_W$-$P$ correlation such proceeding should have heuristic value in analysing the deviations from the correlation. Data for the quantity $\mathbf{R}_P$ are therefore presented in Table 1 and fig. 3.[5]

The curves of fig. 3 have certain features of considerable interest. In the case of the Sudan grass, after harvest 4, $\mathbf{R}_P$ is less the greater the treatment; furthermore, the curves for the plants with higher nitrogen treatment remain relatively constant throughout; the curve with treatment I on the contrary rises continuously after harvest 4. In the case of the oats, the state of affairs is similar from the point

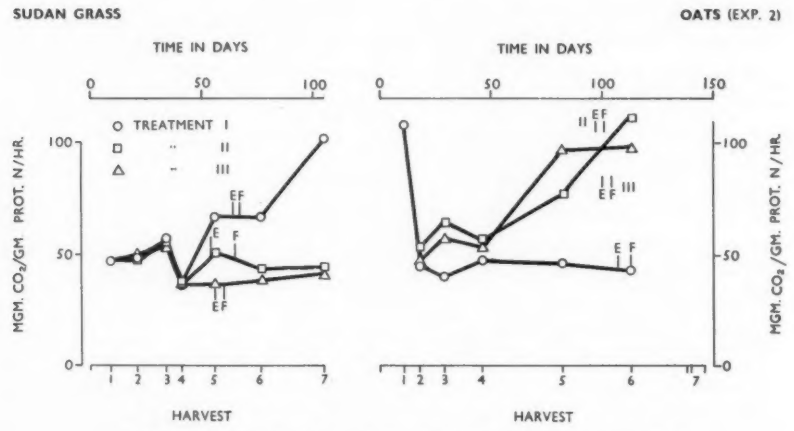



**Figure 3.** Respiration rate per unit of protein-nitrogen ($\mathbf{R}_P$).

of view of the nitrogen status of the plants, those with higher phosphorus supply here being nitrogen deficient; after harvest 4, $\mathbf{R}_P$ increases with treatment and the curve with treatment I remains constant, while the others rise with time.

Gregory and Sen also found that $\mathbf{R}_P$ was increased by nitrogen deficiency. Richards found little effect of phosphorus treatment on $\mathbf{R}_P$ but his high phosphorus leaves had not passed into a nitrogen-deficient phase.

There are several ways in which variations in $\mathbf{R}_P$ may be interpreted.[6] Assuming that both storage and cytoplasmic proteins are present in the leaves, their ratio may be constant in those plants in which $\mathbf{R}_P$ remains constant; and the ratio

[5] Error may have been introduced into the $\mathbf{R}_P$ values by the fact that $\mathbf{R}_W$ relates to tissue that had been for a certain period at 10°C., while $P$ is relevant to the environmental temperature on the day of harvest; it is not known whether appreciable protein hydrolysis took place at 10°C., nor to what extent this might have accounted for the decline in $\mathbf{R}_W$ with time.

[6] Cytoplasm content is not exactly analogous to the protoplasmic factor of Kidd, West and Briggs, which is the effective amount of respiring cell matter. Variations in the protoplasmic state could vary the effectivity without altering the mass of cytoplasm, and such variations could account for variation in $\mathbf{R}_P$.

may be smaller the higher the level of the $\mathbf{R}_P$ curve, i.e. the more nitrogen-deficient the plant; this explanation has already been suggested for the results of Gregory and Sen by Richards and Templeman (1936). Where $\mathbf{R}_P$ rises in senescence this could be interpreted as due to a decreasing ratio of storage to cytoplasmic proteins with time: it is in the nitrogen-deficient plants that greatest hydrolysis of storage proteins would be expected.

The falls in $\mathbf{R}_W$, which occurred during the individual measurements, and which are expressed in fig. 4, show that $\mathbf{R}_W$, although measured at 10° C., did not represent the respiratory index. The curves of fig. 4 suggest that respiration was limited, especially during the early stages of growth, by the concentration of substrates,

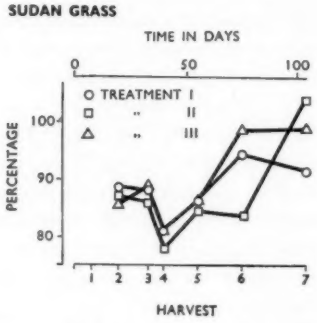



**Figure 4.** Respiration rate on a dry weight basis at 6 hours after cutting expressed as a percentage of its value at 3 hours after cutting. Analysis of variance did not reveal significant interaction of time and treatment, but successive harvest means after harvest 3 were significantly different.

and that the latter increased in the later stages of growth. Gregory and Sen (1937) give the percentage decreases in respiration rate over two consecutive three-hour periods, and it is possible to show that these are correlated with the initial concentrations of total and reducing sugars when the latter are expressed per unit of protein nitrogen. With decreases greater than 10 p.c. the sugar values on this basis are very low, whereas high sugar values are associated with relative constancy of $\mathbf{R}_W$. Caldwell and Meiklejohn (1937) also suggest that oxygen uptake by very young plants is limited by the amount of available respiratory substrate. There is finally considerable evidence, which will be discussed in a subsequent paper from this laboratory by Mrs. J. E. Walkley, that sugar content of leaves is low during active growth, and rises in senescence.[7] Comparison of figs. 3 and 4 suggests that changes in concentration of respiratory substrates may contribute to the drifts of $\mathbf{R}_P$, except in the later stages in the case of the plants of high nitrogen status.

[7] Unfortunately in the present experiments material was insufficient for sugar estimations.

If carbohydrate content satisfactorily explained the variations in $\mathbf{R}_P$, the protein-nitrogen content would become a useful basis upon which to express respiration rate, and would appear to be a sufficiently close measure of the cytoplasm content of the leaves. It must be recalled, however, that Gregory and Sen and Richards believe that the $\mathbf{R}_W$-$P$ correlation indicates an interrelation of respiration and protein metabolism. Gregory and Sen suggest that there is a cycle of protein synthesis and hydrolysis, these two processes proceeding along different paths, catalyzed by different enzymes, and $\mathbf{R}_W$ being a measure of the rate of traverse of the cycle. Gregory and Sen object to the alternative view, that synthesis and hydrolysis take place on the same path, on the grounds that "the relative concentrations of protein and amino-acid would tend to constant values, and an increase in amino-N relative to protein-N could only result from an increase in concentration of the stage previous to amino-acid formation". This objection would not apply to the system suggested by Petrie and Wood (1938); the essence of this system is that at the steady state the concentration of proteins is higher than that of chemical equilibrium, the state being maintained by a continuous expenditure of energy. A fall in the rate of this expenditure is sufficient to account for an increase in amino-acid concentration, which, as various workers have found, appears to cause increase in $\mathbf{R}_W$. Richards, on the basis of remarkable constancy of $\mathbf{R}_P$ in his data, considers that there must be a reciprocal relation between protein and respiration rate, and suggests that to maintain a given protein level a certain minimal rate of respiration may be necessary, and that fall in rate below this value may result in protein hydrolysis.

Evidently there is need for further investigation of the $\mathbf{R}_W$-$P$ relation, especially in structurally uniform material such as that used by Petrie and Wood. For the present the $\mathbf{R}_P$ conception, by abstracting from the effect produced on $\mathbf{R}_W$ by the varying deposition of non-living matter in the cells, enables us to advance the analysis of respiration determinants; and it seems probable that $P$ is a good enough measure of cytoplasm content of the leaves to indicate that variations in this quantity probably determine to a considerable degree not only the decline of $\mathbf{R}_W$ with time, but also treatment effects thereon, including the intersection of the curves in the oats experiment.

## SUMMARY.

An account is given of the temporal drift in respiration rate of the total leaves in two of the experiments described in previous papers of this series. In one of these experiments Sudan grass was grown with varying nitrogen treatments, and in the other oats were grown with varying phosphorus treatments.

On a dry weight basis the respiration rate had an early maximum, after which it declined continuously with time. Increasing nitrogen treatment caused a general increase in rate. Up to a point increased phosphorus treatment caused an

increase in rate during the early growing period; but this effect was subsequently reversed, just as was previously found in the case of protein-nitrogen content.

Analysis of the data leads to the conclusion that not only the decline in respiration rate with time, but also the treatment effects thereon, are largely the result of variations in the cytoplasm content of the leaves; it is also concluded that in data such as the present, extending over a wide range of ontogenetic state, the content of cytoplasm is probably roughly measured by the protein content of the leaves. There is evidence, however, that other factors, possibly including concentration of respiratory substrates, influenced the respiration rates observed.

## REFERENCES.


Ballard, L. A. T. and Petrie, A. H. K. (1936): Austral. J. exp. Biol., 14, p. 135.

Block, R. J. (1934): J. Biol. Chem., 104, pp. 343 and 347; 105, pp. 105 and 455.

Caldwell, J. and Meiklejohn, J. (1937): Ann. Bot., N.S., 1, p. 477.

Chatterjee, U. N. (1933): Bull. Acad. Sci., United Prov. Agra. and Oudh. Allahabad, India, 2, p. 179.

Chibnall, A. C. (1923): J. Biol. Chem., 55, p. 333.

Chibnall, A. C. (1924): *Ibid.*, 61, p. 303.

Chibnall, A. C. (1926): J. Amer. Chem. Soc., 48, p. 728.

Chibnall, A. C. (1933): Biochem. J., 27, p. 1879.

Chibnall, A. C. and Grover, C. E. (1926): Biochem. J., 20, p. 108.

Davies, W. L. (1926): J. Agric. Sci., 16, p. 280.

Foreman, F. W. (1928): J. Agric. Sci., 28, p. 135.

Giroud, A. (1928): Compt. rend. Soc. biol., 98, p. 376.

Giroud, A. (1929): Protoplasma, 7, p. 72.

Gregory, F. G. (1937): Ann. Rev. Biochem., 6, p. 557.

Gregory, F. G. and Richards, F. J. (1929): Ann. Bot., 43, p. 119.

Gregory, F. G. and Sen, P. K. (1937): Ann. Bot., N.S., 1, p. 521.

Hamner, K. C. (1936): Bot. Gaz., 97, p. 744.

Inamdar, R. S., Singh, S. B., and Pande, T. D. (1925): Ann. Bot., 39, p. 281.

Jones, W. W. (1936): Plant Physiol., 11, p. 565.

Joyet-Lavergne, P. (1928): Compt. rend. Soc. biol., 98, p. 501.

Joyet-Lavergne, P. (1929): Protoplasma, 6, p. 84.

Joyet-Lavergne, P. (1932): Compt. rend. Soc. biol., 110, p. 918.

Joyet-Lavergne, P. (1937): Protoplasma, 29, p. 99.

Kidd, F., West, C. and Briggs, G. E. (1921): Proc. Roy. Soc., B, 92, p. 368.

Kiesel, A., Belozersky, A., Agatov, A., Buvschick, N. and Pavlova, M. (1934): Zeitschr. f. physiol. Ch., 226, p. 73.

Loeb, J. (1895): Arch. ges. Physiol., 61, p. 583.

Lyon, C. J. (1924): J. gen. Physiol., 6, p. 299.

Miller, E. J. (1935): Biochem. J., 29, p. 2344.

Mothes, K. (1932): Biol. Centr., 52, p. 193.

Needham, J. (1927): Protoplasma, 1, p. 267.

Nightingale, G. T., Schermerhorn, L. G. and Robbins, W. R. (1928): New Jersey Agric. Exp. Sta. Bull. 461.

Palladin, V. I. (1896): Rev. gén. Bot., 8, p. 225.

Palladin, V. I. (1918): Plant Physiology. English Edition by B. E. Livingston, Philadelphia.

Petrie, A. H. K. (1937) : Austral. J. exp. Biol., 15, p. 385.
Petrie, A. H. K. and Wood, J. G. (1938) : Ann Bot., N.S, 2, p 33.
Phillis, E. and Mason, T. G. (1937) : Nature, 140, p. 370.
Richards, F. J. (1938) : Ann. Bot., N.S., 2, p. 491.
Richards, F. J. and Templeman, W. J. (1936) : Ann. Bot., 50, p. 367.
Singh, B. N. (1928) : J. Ind. Bot. Soc., 7, p. 17.
Smirnow, A. J. (1928) : Planta, 6, p. 687.
Smirnow, A. J. and Izwoschikow, W. P. (1930) : Biochem. Zeitschr., 228, p. 329.
Suzuki, H. (1935) : J. Coll. Agric. Tokyo Imp. Univ., 13, p. 235.
Thomas, W. (1927) : Plant Physiol., 2, pp. 55 and 67.
Williams, R. F. (1936) : Austral. J. exp. Biol., 14, p. 165.
Williams, R. F. (1938) : *Ibid.*, 16, p. 65.
Wood, J. G. and Petrie, A. H. K. (1938) : Ann. Bot. N.S., 2, p. 729.
Zalenski, V. (1902-23) : Summarized by Maximov, N. A., The Plant in Relation to Water. English Translation, London, 1929.

ERRATUM IN PAPER 3.

(Petrie, 1937)

*p. 389, line 14. Delete* "I and".

# COMPARATIVE STUDIES OF *SALMONELLA PULLORUM* AND *SALMONELLA GALLINARUM*

by

J. A. DUMARESQ

(McMaster Animal Health Laboratory, Council for Scientific and Industrial Research, University of Sydney).

*(Submitted for publication 24th October, 1938.)*

*Salmonella gallinarum* was originally described by Klein in 1889 under the name of *Bacterium gallinarum*, and *Salmonella pullorum* by Rettger in 1899, who called it *Bacterium pullorum*. Differentiation between these two organisms has been based on age incidence and clinical features in affected birds, and on certain cultural characters. It was thought for many years that *S. gallinarum* was the exclusive cause of the disease of adult birds known clinically as fowl typhoid, while *S. pullorum* was confined to the disease of chickens generally known as bacillary white diarrhoea or "pullorum disease". The latter was known to be transmissible through the egg. However, in 1912 Jones reported an outbreak of *S. pullorum* infection in adult fowls and since that time similar findings have been recorded from most parts of the world. Recently Bennetts (1935) in Western Australia and Hart and Hungerford (1936) in New South Wales have described outbreaks of *S. pullorum* septicaemia of adult fowls in Australia.

On the other hand, the rôle of *S. gallinarum* in the egg and infections of young chickens has not been so generally recognized. Beaudette (1925) reported the isolation of *S. gallinarum* from outbreaks of disease in chickens 1–3 weeks old, but the identification of the organism was based on fermentation tests in serum water which makes reactions with some carbohydrates unreliable (see below). At the Eleventh International Veterinary Congress, Beaudette (1930) reported having isolated *S. gallinarum* from a dead chick embryo in the egg, and at the same time claimed that about one-third of the outbreaks in young chicks in the State of New Jersey, U.S.A., were due to *S. gallinarum*, but methods used in identification were not given. The same author quotes Beach and Davis as describing an acute outbreak of fowl typhoid in baby chicks 60 hours old. Wagener (1934) refers to the experiments of Martinaglia (1929) and Vizy (1931) and the artificial infection of chickens with *S. gallinarum* in support of the claim that this organism can infect both chickens and adults. Truche, Staub and Bauche (1934) examined 180 affected fowls and chickens with the following results:

| | *S. pullorum.* | *S. gallinarum.* | Miscellaneous Bacteria. |
|---|---|---|---|
| Chickens | 59 | 33 | — |
| Adult fowls | 5 | 75 | 9 |

From the literature it would appear that although *S. gallinarum* predominates in infections of adult fowls and *S. pullorum* in those of chickens, each may occur either in adults or chickens and the diseases produced by the two organisms are indistinguishable in either age group.

Although infections with *S. pullorum* and *S. gallinarum* are now recognized as clinically indistinguishable, it is still claimed that these two organisms can be differentiated by cultural

characters. The literature contains many references to their action on media containing carbohydrates and there is much disagreement concerning the substances which are or are not fermented. A number of subdivisions or intermediate types have been introduced to fit the results obtained by different workers; e.g. *S. pullorum A*, *S. pullorum B*, intermediate type, etc.

It is now generally considered that gas formation in media containing fermentable carbohydrates is not a characteristic of all strains of *S. pullorum*, and that *S. gallinarum* does not form gas, although recently Pacheco and Rodrigues (1936) have recorded a gas-producing strain of *S. gallinarum* which they received from Professor Miessner of Hannover. These authors further state that the gas-producing *gallinarum* strains were first described by Beck and Eber (1929) and confirmed by Gunther (1933). Pacheco and Rodrigues (1936) from an extensive review of the literature compiled the following figures for the fermentation of carbohydrates.

Figures represent the number of workers: + indicates fermentation; — no fermentation, and ± slight fermentation. Their intermediate type is omitted.

| | Dextrose | Galactose | Iso-dulcite | Mannite | Sucrose | Dextrin | Dulcite | Laevulose | Mannose | Xylose | Arabinose | Maltose | Lactose | Raffinose | Sorbite |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S. pullorum* | + | + | + | + | — | ±(2)<br>—(2) | ±(2)<br>—(8) | + | + | +(6)<br>—(2) | + | +(1)<br>±(2)<br>—(10) | — | — | — |
| *S. gallinarum* | + | + | + | + | — | ±(2)<br>+(6)<br>—(2) | +(10)<br>±(1)<br>—(1) | + | + | +(7)<br>—(1) | +(8)<br>±(1)<br>—(1) | + | — | — | ±(1)<br>—(4) |

These authors themselves studied 38 strains from Europe, U.S.A. and South America with the following results:

| | Maltose. | Dulcite. | Dextrin. | Xylose. | Sorbite. | Sodium Potassium Tartrate. |
|---|---|---|---|---|---|---|
| *S. gallinarum* | + | + | — | + | — | + |
| *S. pullorum* | — | — | — | + | + | — |
| Intermediate | + | + | — | — | + | — |

They also report more rapid fermentation of isodulcite (rhamnose) by *S. pullorum* than by *S. gallinarum*, a feature which is also mentioned by Beaudette. Te Hennepe and de Gier (1935) record the fermentation of dulcite by 79 out of 80 Dutch strains of *S. pullorum* but by none of 11 English strains. Mallmann (1931) states that sodium dextro-tartrate is fermented by *S. gallinarum* but not by *S. pullorum*, and that sodium laevo-tartrate is fermented by *S. pullorum* but not *S. gallinarum*.

A more recent differential culture medium is that of Truche, Staub and Bauche (1934), who used peptone agar, to which is added 0·5 p.c. starch, and 1·5 p.c. of a 0·4 p.c. alcoholic solution of bromthymol blue. Slopes of this medium, which is green at the pH of about 7·0, turn blue (alkaline) when sown with *S. gallinarum* and yellow (acid) when sown with *S. pullorum*.

Naidu (1935) claims to have obtained a bacteriophage which will lyse *S. pullorum* but not *S. gallinarum*.

Many observers claim that litmus milk is turned acid by both *S. pullorum* and *S. gallinarum* but later a strong alkaline reaction develops with *S. gallinarum* but not with *S. pullorum*. These, however, are not recognized as constant characters by all workers.

Pacheco and Rodrigues were able to detect differences in the colonial appearance of their groups. They state that on agar non-gas producing strains of *S. pullorum* produce very thin colonies of slow development but that gas-producing strains were more exuberant in their growth

and could not be distinguished from *S. gallinarum* or the intermediate type. In Mallmann's medium, which contains 1: 200,000 brilliant green they claim that *S. pullorum* colonies are small, punctiform, uncoloured, hyaline or opalescent and capitate, while those of *S. gallinarum* are medium sized to large, convex and brilliant. Gassner's medium, which consists of lactose agar and two associated dyes "Wasserblau" and "Metachromgelb", is claimed to give the following results by these workers:

*S. pullorum* (gas producing): slightly yellow colonies.
(non-gas producing): colourless or slightly blue colonies.

*S. gallinarum* and Intermediate type: yellow colonies.

The differential characters that have been employed by most workers are the action on maltose, dulcite and dextrin. The irregularity of the results with these substances is probably largely due to differences in technique. Factors influencing the results obtained and which have undoubtedly been overlooked by some workers are:

1. Certain carbohydrates may be partially hydrolyzed during sterilization by heat and it is always preferable to use sugar solutions sterilized by filtration. Dextrin appears to be especially liable to break down to maltose in this way, and it is difficult to obtain samples of dextrin free from traces of this sugar. On the other hand maltose is more stable, and Pacheco and Rodrigues, searching for the cause of the irregular results ascribed to maltose by some workers, autoclaved it at 120°C., but were unable to demonstrate any hydrolysis as the result of such treatment.

2. Unsuitable nutrient substrate is another source of error. Media containing normal unheated serum cannot be used with dextrin or maltose because of the enzymes contained in serum. Spray and Doyle (1921) found that both *S. pullorum* and *S. gallinarum* fermented maltose in a medium of 4 parts horse serum and 1 part water. To this medium 1 p.c. of the agar and Andrade's indicator were added and the whole then autoclaved. It is probable that sufficient time elapsed between the mixing of the various constituents and autoclaving for the enzymes of the raw serum to act upon the maltose. Hendrickson (1927) using a similar medium also records fermentation of maltose, dextrin and dulcite by *S. pullorum*. While serum enzymes would account for the results with dextrin and maltose, they do not act upon dulcite, and the fermentation of this substance by *S. pullorum* may have been due to some other causes, or as indicated by Te Hennepe's observations, it may be that some strains of *S. pullorum* do really ferment this substance. It has not been possible to consult the original article by Te Hennepe, so his methods and media are not known.

Pacheco and Rodrigues (1936) have shown that maltose is broken down to glucose by raw serum and that this action was not inhibited by heating the serum to 60°C. Recently Goldsworthy, Still and Dumaresq (1938) have shown that even starch is converted into glucose by serum enzymes and that both *S. pullorum* and *S. gallinarum* appear to ferment starch and maltose if raw serum is present in the medium.

In their antigenic relationship *S. pullorum* and *S. gallinarum* have been placed together in the same group according to the Kauffmann and White schemes, and have been found to share the same "O" antigens (viz. Kauffman IX(XII)–).

As they are both non-motile there is no possibility of differentiation by "H" antigens. It appears to be generally agreed that no one has convincingly demonstrated any antigenic differences between the two organisms even by absorption experiments.

Pacheco and Rodrigues prepared antisera against gas-producing and non-gas producing strains of *S. pullorum* and *S. gallinarum* as well as the type which they class as intermediate. They were unable to detect any significant difference between the types by absorption and cross-agglutination tests. Hendrickson was also unable to show any antigenic differences in similar experiments.

## PRESENT INVESTIGATIONS.

Observations have been made on three strains of an organism isolated from a septicaemic disease of fowls occurring in Tasmania and clinically resembling fowl typhoid, six strains of *Salmonella pullorum* isolated in New South Wales and one from Tasmania, and a stock strain each of *S. pullorum* (No. 970) and *S. gallinarum* (No. 416) obtained from the National Collection of Type Cultures, Lister Institute, London.

As will be seen below the three unknown Tasmanian strains conformed with the characters usually ascribed to *S. gallinarum*. There is no record of the occurrence of this organism in Australia, and this fact stimulated an enquiry into the relationship of *S. gallinarum* and *S. pullorum*.

## CULTURAL CHARACTERS.

*Colony type:* After 18 hours' incubation at 37°C. all strains showed circular, low conical colonies, 1–2 mm. in diameter, with entire or slightly crenate edges. On further incubation there was an increase in size to about 5 mm. diameter after 48 hours, by a flat spreading growth of the periphery. The appearances of colonies in the latter stages of growth varied, some developing a rough, irregular or papillated surface and crenated edges, while others remained smooth.

TABLE 1.

| Organism. | Dextrose. | Lactose. | Maltose. | Duleite. | Dextrin. | Sorbite. | Xylose. | Iso-duleite (Rhamnose) | Arabinose. | Mannite. | Sodium mucate. | Sodium d-tartrate. | Sodium potassium tartrate. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S. gallinarum* F1 | A | — | A | A | sl A | A | A 5d | A 7d | A | A | sl A —7d | sl A 3d —7d | sl A 4d —8d |
| *S. gallinarum* F2 | A | — | A | A | sl A | A | A 6d | A 7d | A | A | sl A —7d | — | sl A 4d —8d |
| *S. gallinarum* F3 | A | — | A | A | sl A | A | A 4d | A 7d | A | A | sl A —7d | — | sl A 4d —8d |
| *S. gallinarum* (NCTC 416) | A | — | A | A | sl A | A | A 2d | A 4d | A | A | sl A —7d | sl A 2d —7d | sl A 4d —8d |
| *S. pullorum* (NCTC 970) | A | — | — | — | — | A | A17d | A | A | A | — | — | — |
| *S. pullorum* 611 | A | — | — | — | — | A | A19d | A | — | — | — | — | — |
| *S. pullorum* 1380 | A | — | — | — | — | A | A 2d | A | — | — | — | — | — |
| *S. pullorum* 1045 | A | — | — | — | — | A | A 2d | A | — | — | — | — | — |
| *S. pullorum* 1132 | A | — | — | — | — | A | A17d | A | — | — | — | — | — |
| *S. pullorum* 2824 | A | — | — | — | — | A | A 2d | A | — | — | — | — | — |
| *S. pullorum* 2958 | A | — | — | — | — | A | A 2d | A | — | — | — | — | — |

A = acid developing within 24 hours. A 4d = acid developing in 4 days.

*Morphology:* All strains appeared like typical *Salmonella* under the microscope—Gram negative rods or coccobacilli with considerable pleomorphism.

*Carbohydrate Fermentation:* Tests were carried out in peptone water containing 1 p.c. carbohydrate plus Andrade's indicator. The carbohydrates were made up in concentrated solutions,

sterilized by filtration and stored in sealed ampoules till required. A sufficient volume to make a 1 p.c. solution was added to the peptone water immediately before use. Each tube was sown with several drops of a V.F.[1] broth culture, and incubated for at least three weeks. The results are shown in Table 1.

Macerate at 50°C. for 24 hours then heat to 80°C. for few minutes. Stand overnight, decant and filter through moistened filter paper. Heat to 90°C. and alkalinize with NaOH. Heat to 120°C. for ½ hour to precipitate phosphates. Filter and sterilize.

Reliance cannot be placed on the results obtained with dextrin because of the frequent presence of small traces of maltose. Where only a slight acidity was produced this usually disappeared in about a week. This was probably due to alkali produced by the breakdown of the peptone, as it was found that these organisms produced an alkaline reaction in plain peptone water about this time. (Tested by growing the organisms in peptone water plus bromthymol blue.) All strains of *S. pullorum* produced gas in dextrose and in some of the other sugars.

The results indicate that maltose, dulcite, sodium mucate and sodium potassium tartrate are fermented by *S. gallinarum* but not by *S. pullorum*. This is in agreement with the results obtained by Pacheco and Rodrigues, but it has not been possible to confirm Mallmann's results with sodium dextro-tartrate entirely, and this experiment would indicate that this substance is not to be relied upon. It is possible that acid production from it is so feeble and transitory as to be very easily missed.

*Litmus milk:* All strains tested (four *S. gallinarum* strains and one stock *S. pullorum*) produced a slight initial acidity within 48 hours, which even with *S. pullorum* tended to return to neutral in about 2 weeks but with *S. gallinarum* a strong alkaline reaction resulted in 2–4 weeks.

All strains produced $H_2S$ in lead acetate agar and reduced nitrates to nitrites when tested by Ilosvay's method.

Voges-Proskauer and Methyl Red tests were negative and there was no production of indole by the strains tested.

*Bromthymol blue Agar:* The first medium of this nature that was tried was that recommended by Truche, Staub and Bauche (1934), containing 0·5 p.c. starch and 1·5 p.c. of a 0·4 p.c. alcoholic solution of bromthymol blue. These substances were added to ordinary peptone agar. At pH about 7·0 this medium is green, while an alkaline reaction turns it blue and an acid one yellow. Slopes of this medium sown with young broth cultures of the organisms under examination gave the following results:

*S. gallinarum:* Sloped portion blue, butt yellow, later turning blue.
*S. pullorum:* Whole of medium yellow.

On prolonged incubation those tubes sown with *S. pullorum* may return to a green colour or even become slightly blue, but when read after 24–48 hours' incubation, the results are always clear-cut. We have lately omitted the starch without in any way affecting the results, and now only add the requisite amount of bromthymol blue to ordinary peptone agar.

Thirty-three strains of *Salmonella*, including members of all serological groups, have been tested on this medium. All of these gave a blue colour corresponding to that produced by

[1] *V.F. Broth:* This is prepared as follows: Ox meat, 500 gm. (minced); ox liver, 500 gm. (minced); pig stomach, 600 gm. (minced); tap water, 5 litres; HCl, 50 c.c.

*S. gallinarum* except *S. typhi suis* and *S. typhi suis v. Voldagsen* which gave a yellow-green colour corresponding to *S. pullorum*. *B. coli* also gave a blue colour though that produced by *B. lactis aerogenes* was yellow.

A stab culture of *S. pullorum* in this medium gave a yellow colour while that of *S. gallinarum* was yellow with a narrow blue zone at the top (probably the aerobic portion of the medium). If incubated anaerobically both organisms gave a yellow colour. It should be noted that V.F. agar must not be used in this test as it leads to very irregular results.

### *Serological Comparisons.*

Several strains of both *S. gallinarum* and *S. pullorum* were tested for "smoothness" by agglutination tests with a rough *S. anatum* antiserum. Finally *S. gallinarum* F3 and *S. pullorum* 611 were selected as being "smooth", both by colonial morphology and agglutination tests. Occasionally either of these strains would give slight agglutination deposits without visible clearing of the suspension, in all titres with *S. anatum* serum but never more, and this was interpreted as being due to slight differences in the sensitivity of the antigen rather than an indication of serological variation.

Three different antigens were prepared from each strain:

(1) *Formalized antigen:* Grown on V.F. agar in flat bottles for 18 hours, washed off with sterile normal saline. Sterilized by the addition of 0·25 p.c. formalin and incubation for 2 days at 37°C.

(2) *Steamed antigen:* Grown and suspended in saline as (1). Sterilized by steaming for 1 hour.

(3) *Live antigen:* Grown on V.F. agar for 18 hours and suspended in sterile normal saline. Prepared freshly each time required.

*Preparation of antisera:*

Antisera against *S. pullorum* and *S. gallinarum* were prepared with each of the above antigens. Sheep were inoculated intravenously with increasing doses of antigen at weekly intervals until serum from a trial bleeding indicated that a satisfactory titre had been reached. All sera were preserved with 0·5 p.c. carbolic acid.

*Cross Agglutination Tests:*

Cross agglutination tests were set up between each antiserum and antigen. Dilutions in geometrical progression were carried out with 0·25 p.c. formol saline, except those in which live antigens were used in which case sterile normal saline and sterilized and plugged agglutination tubes were used. All tests were read after 24 hours' incubation at 37°C.

The titre of the serum in most cases was 1: 1600 or 1: 3200. The highest was that of the *S. gallinarum* antiserum prepared with live organisms which agglutinated live *S. pullorum* and *S. gallinarum* antigens to a titre of 1: 6400.

There was no appreciable difference between *S. pullorum* and *S. gallinarum* when tested by cross agglutination with the different antigens and antisera used in these experiments.

### *Agglutinin Absorption Tests.*

As no differences were revealed by cross-agglutination it was decided to try agglutinin absorption tests with *live* antigens, and antisera prepared against these,

although numerous other workers using dead antigens have failed to find any difference between the two organisms.

Absorbing antigens were prepared by growing the organisms on 9 cm. petri dishes of V.F. agar for 18 hours and washing off the growth with sterile saline. The suspensions were then washed twice with sterile saline to remove traces of medium and finally suspended in 6·25 c.c. of a 1/12·5 dilution of the antiserum to be absorbed. The absorbing dose was the growth of three agar plates per 0·5 c.c. of antiserum, and absorption was carried out for a minimum of two days at refrigerator temperature (about 4°C.). At the end of this time the suspensions were centrifuged and the absorbed antisera drawn off.

The results of cross-agglutination tests of these absorbed sera with live antigens showed that all agglutinins had been absorbed from the *S. gallinarum* antiserum, but that although the *S. gallinarum* F3 suspension removed all agglutinins from its homologous antiserum, *S. pullorum* antiserum absorbed with this still showed +[2] agglutination with *S. pullorum* antigen in dilutions from 1:25 to 1:100.

As the test with *S. pullorum* antiserum absorbed with *S. gallinarum* antigen seemed to be the only one pointing to any distinction in antigenic structure between the two organisms, this possibility was examined further.

Another batch of *S. pullorum* antiserum was absorbed with live *S. gallinarum* as before, but the absorbing dose was increased to 4 plates per 0·5 c.c. of antiserum. This absorbed serum was tested against four strains of *S. pullorum*, using live antigens as before.

All *S. pullorum* antigens (Nos. 611, 1380, 976A and F26) showed + agglutination at 1:25 and Nos. 611 and 1380 a trace at 1:50. *S. gallinarum* F3 antigen showed only a trace at 1:25. All the above antigens were checked for smoothness with *S. anatum* antiserum and found negative at 1:25.

Since this experiment still indicated that absorption with *S. gallinarum* F3 failed to remove all trace of agglutinins for *S. pullorum* from an *S. pullorum* antiserum, another strain of *S. gallinarum* was tried.

Two batches of *S. pullorum* 611 antiserum, one prepared against live and the other against formalized antigen, were each absorbed with *S. gallinarum* F2 live antigen, using 4 plates per 0·5 c.c. of antiserum as above. These two absorbed sera were tested against live antigens of different strains of *S. pullorum*. *S. pullorum* 611 antiserum prepared with live antigen and absorbed with live *S. gallinarum* F2 antigen gave + agglutination at 1:25 with two of the *S. pullorum* antigens (Nos. 1380 and 17), but no agglutination with Nos. 611, 1103, or *S. gallinarum* F2.

*S. pullorum* 611 antiserum prepared with formalized antigen and absorbed with live *S. gallinarum* F2 antigen gave traces of agglutination with all the antigens at 1:25 dilution and also a trace at 1:50 with *S. pullorum* antigens 611 and 1380.

As the results of these experiments, though not conclusive, suggested the possibility of *S. pullorum* possessing a small amount of an antigen which was not shared with *S. gallinarum*, the antiserum was fractionated in an attempt to concentrate the antibodies.

---

[2] + = agglutination without visible clearing. ++ = agglutination with appreciable clearing. +++ = complete agglutination.

This was done by precipitating the euglobulin fraction of the *S. pullorum* serum which was then concentrated by evaporating over $CaCl_2$, in a dessicator, under reduced pressure, from a volume of 40 c.c. to 6·5 c.c.

The titre of this concentrated serum was 1: 12,800. This was absorbed with live washed *S. gallinarum* F3 antigen as before. The antigens used were live suspensions of *S. gallinarum* F3 and *S. pullorum* 1380 washed with saline. There was no agglutination of the former, but the *S. pullorum* 1380 antigen was agglutinated ++ at 1: 25 with a trace at 1: 50.

It will be seen that though the results still suggest that *S. pullorum* may possess a small amount of an antigen which *S. gallinarum* does not contain, they are probably not sufficiently clear-cut to justify a claim to any antigenic difference between *S. pullorum* and *S. gallinarum*, as judged by somatic cross-agglutination and agglutinin absorption.

*Test for Vi Antigen.*

Another method tried in the analysis of the antigenic pattern of these two organisms was an attempt to detect Vi agglutinins according to the method described for *Salmonella* by Felix and Pitt (1936).

*S. pullorum* 611 and *S. gallinarum* F3 antisera, prepared against live antigens were each absorbed with their homologous antigen as in previous experiments, except that the antigens were previously treated with 5 p.c. HCl at room temperature for two days to destroy Vi antigen if present. The antisera were then tested for residual agglutinins against live and acid treated homologous antigens.

In all cases the results were completely negative, showing that the strains used possessed no Vi antigens detectable by this method. As they had previously been maintained on laboratory media for some time, and as no virulence tests were conducted on birds or animals, it is possible that these organisms may possess Vi antigens when acting as natural pathogens in fowls.

## SUMMARY.

(1) Comparative cultural studies of four strains of *S. gallinarum* and eight strains of *S. pullorum* have been carried out.

(2) A brief review with discussion of some relevant literature is given.

(3) The strains of *S. gallinarum* and *S. pullorum* studied could be differentiated by the fermentation of maltose, dulcite, sodium mucate and sodium potassium tartrate by the former and not the latter. Also by their reactions in litmus milk and bromthymol blue agar.

(4) No significant serological differences could be detected by cross agglutination or agglutinin absorption.

(5) No Vi antigen could be detected in a single strain examined from each species.

## ACKNOWLEDGMENT.

The writer wishes to acknowledge his indebtedness to Dr. H. R. Carne for his great help in the bacteriological and serological studies.

## REFERENCES.

Beaudette, F. R. (1925): J. Amer. vet. med. Ass., 67, p. 741.
Beaudette, F. R. (1930): 11th International veterinary congress, Report, 3, p. 705.
Bennetts, H. W. (1935): Austral. vet. J., 11, p. 223.
Felix, A. and Pitt, R. M. (1936): Brit. J. exp. Path., 17, p. 81.
Goldsworthy, N. E., Still, J. L. and Dumaresq, J. A. (1938): J. Path. Bact., 46, p. 253.
Hart, L. and Hungerford, T. G. (1936): Austral. vet. J., 12, p. 17.
Hendrickson, J. M. (1927): J. Amer. vet. med. Ass., 70, p. 629.
Mallmann, W. L. (1931): Proc. Soc. exp. Biol., N.Y., 28, p. 501.
Martinaglia, G. (1929): J. S.Afr. vet. med. Ass., 1, p. 77.
Naidu, P. M. N. (1935): Bull, Acad. vet. Fr., 8, p. 306
Pacheco, G. and Rodrigues, C. (1936): Mem. Inst. Osw. Cruz., 31, p. 591.
Spray, R. S. and Doyle, L. P. (1921): J. infect. Dis., 28, p. 43.
Te Hennepe, B. and De Gier, C. J. (1935): Tijdschr. Diergeneesk., 62, p. 187.
Truche, C., Staub, A. and Bauche, J. (1934): Bull. Acad. vet. Fr., 7, p. 268.
Vizy, L. (1931): Közl. Összehas élét-es-kórtan Köreböl., 24, p. 598.
Wagener, K. (1934): 12th International veterinary congress, Report 3, p. 108.